Aspects of liposome-tumor cell interaction

Renswoude, Anne Joseph Bernardus Maria van

ASPECTS OF LIPOSOME-TUMOR CELL INTERACTION

A.J.B.M. VAN RENSWOUDE

RIJKSUNIVERSITEIT TE GRONINGEN

ASPECTS OF
LIPOSOME-TUMOR CELL INTERACTION

PROEFSCHRIFT

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ANNE JOSEPH BERNARDUS MARIA VAN RENSWOUDE

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PROMOTOR : Dr. G.L. Scherphof

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S T E L L I N G E N

I

De conclusie van Hock en Stöhr, dat de interaktie tussen multilamellaire liposomen en HeLa cellen kan worden gequantificeerd door de overdracht van de lipofiele fluorescerende verbinding perylene tussen liposomen en cellen te meten, berust op onvoldoende experimentele gegevens.

Hock, D. and Stöhr, M. (1977) Histochemistry 52, 97-103.

II

Voorlichting door de dagbladpers omtrent veelbelovende ontwikkelingen in gebieden van fundamenteel medisch-biologisch onderzoek dient te geschieden onder inachtneming van tenminste die mate van soberheid, voorzichtigheid en nauwgezetheid, welke gemeenlijk door de wetenschappelijke vakpers wordt gehanteerd.

De Volkskrant, 18 april 1979.

Weinstein, J.N. et al. (1979) Science 204, 188-191.

III

Het recombinant-DNA onderzoek aan bacteriën levert nu reeds resultaten welke van direkt praktisch belang zijn voor de geneeskunde.

Goeddel, D.V. et al. (1979) Nature 281, 544-548.

IV

Het is soms beter te spreken van "liposomen als carriers voor potentiële geneesmiddelen", dan van "liposomen als potentiële carriers voor geneesmiddelen".

V

Er is vooralsnog geen wetenschappelijke grond voor de wijdverbreide opvatting, dat lezen en manipuleren bij "slecht licht" blijvende schade berokkent aan het gezichtsvermogen.

VI

De suggestie van Nakatsu en Cameron, dat liposomen zouden kunnen dienen als carriers voor "spier-specifieke" geneesmiddelen, is uiterst voorbarig.

Nakatsu, K. and Cameron, D.A. (1979) Can. J. Physiol. Pharmacol. 57, 756-759.

VII

De door Maxam and Gilbert ontwikkelde methode voor de base-sequentie analyse van DNA draagt in hoge mate bij tot een versnelde opheldering van de details der genfunktie.

Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 560-564.

VIII

De term "fusie", ter aanduiding van de aard van een mogelijk mechanisme van liposoom-cel interaktie, dient met voorzichtigheid te worden gebezigd.

Dit proefschrift.

IX

Het "cluster"-hoofdpijn syndroom is, vooral als het zich in een chronische vorm manifesteert, een uiterst invalidiserende aandoening, welke een adequate en zo mogelijk causale therapie behoeft. Bij de medicamenteuze behandeling van de chronische vorm van het syndroom met behulp van ergotamine-preparaten is de kans op het optreden van ergotisme-verschijnselen zeer reëel aanwezig.

Wentges, R.Th.R. (1975) Ned. T. Geneesk. 119, 348-354.

X

Bij de huidige stand van zaken voor wat betreft de voor wetenschappelijk onderzoek beschikbare budgetten, moet het noodzakelijk worden geacht álle wetenschappelijk onderzoek, óók datgene wat in handen is van promovendi, zo goed mogelijk gecoördineerd en vooral zoveel mogelijk in samenwerkingsverband te doen verrichten.

XI

De enig juiste en afdoende behandeling van panaritia is de chirurgische.

Verschueren, R.C.J. (1974) Ned. T. Geneesk. 118, 1249-1254.

XII

De aansporing: "weest vruchtbaar en vermenigvuldigt" ontbeert helaas elke aanduiding van een vermenigvuldigings*factor*.

Genesis 1 : 28 (Statenvertaling).

Stellingen behorende bij het proefschrift "Aspects of liposome-tumor cell interaction".

A.J.B.M. van Renswoude 19 december 1979

Voor Hanneke,
Stephanie en Frederieke

C O N T E N T S

V O O R W O O R D

Het in dit proefschrift beschreven onderzoek werd grotendeels verricht in het laboratorium voor Fysiologische Chemie van de Rijksuniversiteit te Groningen. Velen hebben bijgedragen aan de verwezenlijking van deze dissertatie. Mijn dank gaat allereerst uit naar mijn echtgenote Hanneke, en onze kinderen, voor het begrip en de morele steun die zij mij tijdens het onderzoek hebben gegeven. Mijn promotor Gerrit Scherphof wil ik graag bedanken voor de heldere wijze waarop hij het onderzoek begeleid en het uiteindelijke manuscript van kritische kanttekeningen voorzien heeft. Mijn copromotor Ab Kroon dank ik voor het zorgvuldig doornemen van het manuscript en voor de vele plezierige gedachtenwisselingen. Prof. Loomeijer ben ik zeer erkentelijk voor zijn aanmoediging destijds om met een promotie-onderzoek te starten. Voorts wil ik mijn dank betuigen aan: Henk Westenberg, die mij met zijn grote routine en gevoel voor het vak op een vaak zeer plezante wijze heeft bijgestaan op het experimentele vlak; Ton Konings voor zijn interesse in het onderzoek en de goede samenwerking in een aantal proeven; Dick Hoekstra voor de vele nuttige discussies en het recente hechte teamwork in de frontlinie; Harma Ellens, Peter Verheijen, Henk Wolters, Frits Roerdink, Jan Wilschut, Siebrand Poppema, Jan Weening, Caesar Hulstaert, Bert Meijer, en alle niet met name genoemden, die op enigerlei wijze een bijdrage hebben geleverd. I am indebted to Dr. Robert Blumenthal and Dr. John Weinstein (National Cancer Institute, N.I.H., Bethesda, MD, U.S.A.) for many fruitful discussions and for their hospitality during my visit to their laboratory in december 1978. I consider it an honor and a pleasure to join their group next spring. Je remercie Dr. F. Zajdela (Faculté des Sciences, I.N.S.E.R.M., Orsay, France) pour mettre à ma disposition les cellules cancéreuses qui portent son nom.

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Jos van Renswoude

CHAPTER I

GENERAL INTRODUCTION

When we started our work in 1976, our mandate was: "find an answer to the question how and to what extent liposomes interact with mammalian tumor cells, and see whether such an answer can bring about a rational effort to use liposomes as carriers for antineoplastic drugs". Others must have been operating with similar goals in mind, since during the years to follow the number of publications dealing in some way with the questions stated above grew almost exponentially with time. It would be entirely beyond the rational and physical scope of this thesis to give an concise survey of all relevant literature, especially since we would feel forced to take also into consideration the fast progress in some very closely related fields, e.g. membrane biology and -chemistry, and biology of the cell-surface. A range of studies has been covered by a number of extensive reviews (1-19). In the following we will confine ourselves to a brief description of some fundamental features and -applications of liposomes, and of the possibilities and consequences of liposome-cell interaction.

Liposomes

Liposomes, alternatively called phospholipid vesicles or (lipid)vesicles, are spherically shaped particles which consist of one or more closed concentric phospholipid bilayers (lamellae) surrounding as many aqueous compartments. Within the bilayer the phospholipids are arranged in such a way, that their polar head-groups face the aqueous phase, whereas their apolar hydrocarbon chains are pointing towards the centre of the membrane (see Fig. 1). Large multilamellar liposomes (Fig. 1; top, left) form spontaneously when dry phospholipids are dispersed into excess water (20,21). They are quite heterogeneous with respect to size; on the average their diameter is in the order of several μm. A fundamental feature of liposomes is the basic structural unit they share with biomembranes: the phospholipid bilayer (1). As such, liposomes may be considered abstract forms of biomembranes, because they contain, in contrast to biomembranes, no proteins (for extensive reviews on the structure and function of biomembranes see Refs. 22-29). The basic structural similarity

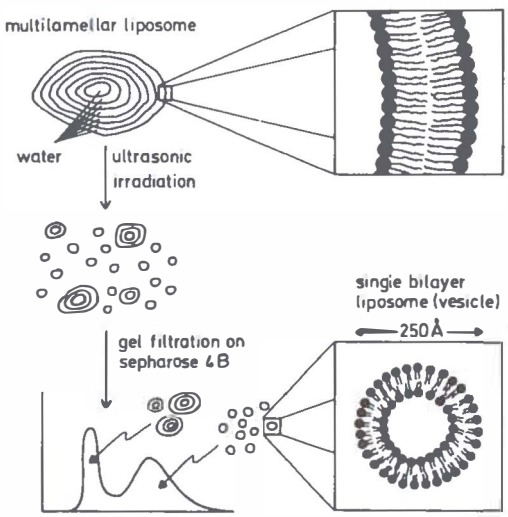


Fig. 1. *Schematic representation of the preparation and appearance of liposomes.*

between liposomes and biomembranes is reflected in some important properties of liposomes. Firstly, their membrane constitutes a permeability barrier for ions, electrolytes, non-electrolytes and macromolecules (3,20). Secondly, liposomal membranes, just like biomembranes, can exist at two different levels of physical order. Depending on the constituting phospholipid species, on temperature, and on the composition of the aqueous phase, the bilayer may be in a gel-state ("solid") or in a liquid-crystalline state ("fluid"). In the "solid" phase, the motional freedom of the phospholipid hydrocarbon chains is highly restricted, resulting in a tight molecular packing of the membrane. In the "fluid" phase the chains are highly mobile ("melted"), as a result of which the thightness of molecular packing in the bilayer is reduced. The temperature at which the transition from one state into the other occurs, is called the phase transition temperature (T_c) and is, inter alia, characteristic for the phospholipid species that constitutes the membrane. In case the bilayer comprises more than one phospholipid species, or when it contains in addition non-phospholipids or non-lipids, its phase behaviour is rather complex (for reviews on the phase-behaviour of liposomes and biomembranes see Refs. 30-32). The basic structural similarity between biomembranes and lipo-

somes make the latter very suitable as model membranes, for the investigation of structure and function of membranes in general, *e.g.* lipid-protein interactions (1,3), transport of molecules across membranes (3) and immunological properties of membranes (9,10,16).

Liposomes can be prepared from a variety of naturally occurring or synthetic phospholipids. Depending on their composition and way of preparation, liposomes differ in charge, size and number of lamellae. The incorporation of amphiphilic lipid-like compounds such as stearylamine or dicetylphosphate into the bilayer imparts positive- or negative charge, respectively, to the liposomes. Negatively charged liposomes can also be obtained upon incorporation of phospholipids which possess a net negatively charged polar head-group, such as phosphatidylglycerol, phosphatidic acid, and phosphatidylserine. Other lipids, like cholesterol, can be accomodated in artificial phospholipid bilayers. Cholesterol, widely occurring in biomembranes, reduces the fluidity of liquid-crystalline bilayers, making them more "rigid" (33), and lowers their permeability for a variety of hydrophilic compounds (see *e.g.* Ref. 3). Cholesterol is believed to play an important role in the regulation of membrane microviscosity and -curvature (34). Liposomes can be prepared by a variety of techniques, most of which render vesicle populations characteristic with respect to average size, size-homogeneity and number of lamellae. Small unilamellar vesicles with a minimum diameter of approximately 25 nm are conveniently obtained (see Fig. 1) by ultrasonic irradiation (= sonication) of multilamellar dispersions (35). Other ways of preparing small unilamellar vesicles involve the use of detergents (36,37), injection of ethanolic solutions of phospholipids into aqueous medium (28,39), and the use of a French press (40). Large unilamellar vesicles, with diameters ranging from 200-1000 nm can be produced by infusion of an etheric solution of phospholipids into warm aqueous medium (41), via fusion of small unilamellar vesicles, consisting of phosphatidylserine (42), or by organic-solvent evaporation from a phospholipid inverted-micelle dispersion (43).

Non-lipid molecules may become associated with liposomes in two principally different ways: by entrapment in the inner aqueous compartment(s) (hydrophilic substances) or by incorporation into the bilayer(s) (lipophilic compounds). Amphipathic molecules other than phospholipids will generally partition between the aqueous phase and the bilayer. In case of charged liposomes, oppositely charged non-lipid molecules may be electrostatically adsorbed to either

side of the bilayer. The amount of hydrophilic molecules that can be entrapped inside liposomes depends on the mode of preparation, on liposome charge, -size and number of lamellae, as well as on the concentration of the compound to be entrapped. The volume of entrapped water ranges from 0.3 - 4 µl per µmole of total liposomal lipid (*cf.* Refs. 43,44). Liposomes carrying encapsulated material can be separated from non-entrapped substance by gel chromatographic techniques (35,45), centrifugation (in case of multilamellar vesicles), or dialysis.

The capacity of liposomes to carry all kinds of lipid- and non-lipid substances has drawn them into the attention of cell-biologists and pharmacologists. It was recognized quite early after their "discovery" by Bangham and colleagues (21) that liposomes could provide valuable tools to modify cellular behaviour by introducing into cells "foreign" molecules which normally would not or insufficiently become cell-associated (46-50). Ever since, a large number of studies has been devoted to *in vitro* liposome-cell interactions as well as to the possibilities of *in vivo* pharmacological applications of liposomes as carriers. *In vivo* studies have mainly focussed on the possibilities of using liposomes in enzyme-replacement therapy of lysosomal storage diseases (reviewed in Ref. 17; see also Ref. 51), cancer chemotherapy (reviewed in Ref. 8), treatment of diabetes (52) and of heavy metal poisoning (53). Furthermore, the possibilities of using liposomes in the treatment of the respiratory distress syndrome (54,55), in the steroid-therapy of arthritis (56,57), as immunological adjuvants (58) and recently, as carriers of antiparasitic drugs (59,60) have been considered. Two important findings have emerged directly or indirectly from *in vivo* investigations: Firstly, upon intravenous injection a considerable fraction of the administered liposomes ends up in the reticulo-endothelial tissues, mainly of liver and spleen (15). Secondly, liposomes interact with plasma components, resulting in exchange of lipids with lipoproteins (61), in leakage of entrapped solutes (62), or in complete disintegration of the liposomes (63). As yet, no conclusive methods have been developed to monitor completely the kinetic fate of liposomes *in vivo*.

Liposome-cell interactions

Liposome-cell interactions, both *in vitro* and *in vivo* may comprise three major, possible mechanisms: Adsorption of liposomes to the cell surface, endocytosis, and fusion between liposomal- and plasma membrane. These mecha-

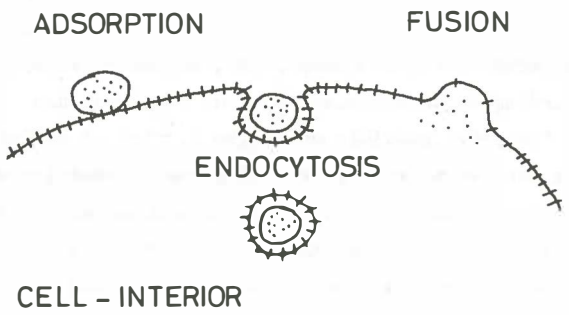


Fig. 2. *Schematic and simplified view of possible liposome-cell interactions.*

nisms are depicted in Fig. 2, in a highly simplified and schematic way. Let us first note, that transfer or exchange of lipid between liposomes and cells (64) may occur with each of the three possible interaction mechanisms mentioned above. If cells and liposomes are brought together in a serum-containing medium, such transfer/exchange of lipid may even take place without physical contact between liposomes and cells; it may be mediated through specific exchange-proteins and other serum components (61). The "simplest" way of liposome-cell interaction involves adsorption of vesicles to the cell surface. Adsorption could be lasting ("stable") or transient. At present there are several lines of evidence in support of such adsorption; most of these studies have been carried out with small unilamellar vesicles consisting of phospholipids in the "solid" state (*e.g.* Refs. 65-67). Presumably, cell-surface proteins are in some way involved in the vesicle-adsorption process (Refs. 65, 68, see also Chapter IV). In case of stable adsorption, the liposome-contents will be gradually released into the surrounding medium (see also Chapter IV). Following adsorption, endocytosis (for a review on endocytotic mechanisms see Ref. 51) may take place: engulfment of the liposome by the plasma membrane leading to ingestion of the liposome as a particle. The resulting endocytotic

vacuole could fuse with a primary lysosome to form a secondary lysosome. Within the latter structure the liposome is expected to be ultimately degraded by lipolytic enzymes (67). Upon endocytosis of liposomes, the fate of liposome-entrapped compounds will depend on their nature; *e.g.* small solutes may leak into the cytoplasm and large molecules such as proteins or nucleic acids could be degraded in the lysosomes. In a number of reports data have been presented according to which endocytosis of liposomes has occurred (*e.g.* Refs. 13,15). The third possible mechanism is that of vesicle-cell fusion. Such fusion will involve molecular intermingling of vesicle- and plasma membrane constituents, and coalescence of the vesicle aqueous interior and the cytoplasm (for reviews on membrane-fusion see Refs. 70,71). Vesicle-entrapped substances will consequently be delivered directly into the cytoplasm. To be able to speak of vesicle-cell fusion one therefore has to demonstrate the primary intracytoplasmic localization or -activity of a formerly liposome-entrapped substance. This criterion has been fulfilled in some studies (66, 72-75, see als Chapter VII). The major part of this thesis describes experiments and considerations on the kinetics and mechanisms of *in vitro* liposome-cell interaction (Chapters II, III, IV and VII).

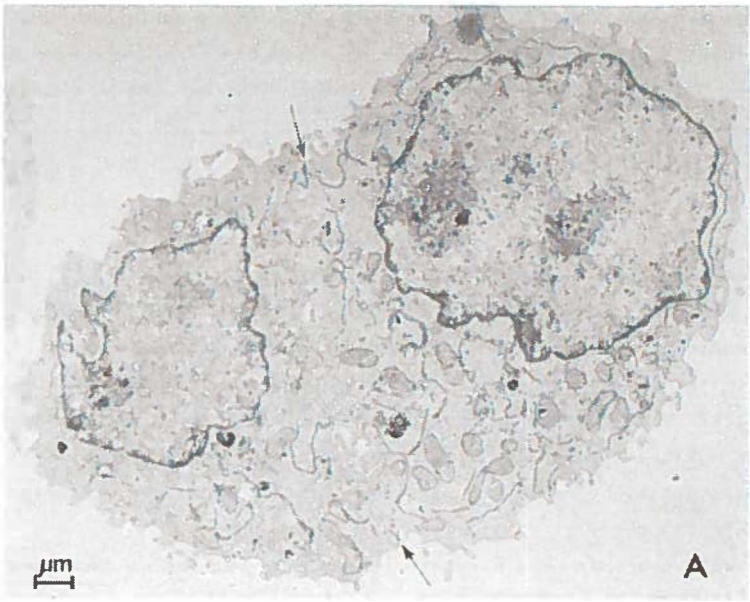
The interest of others and ourselves in the mode and extent of vesicle-tumor cell interactions derives partly from the consideration that liposomes might deliver antitumor agents to neoplastic cells. For example, failure of a tumor cell to respond adequately to an antineoplastic drug (drug-resistance) may result from reduced or absent cellular permeability to the drug. Liposomes could be envisaged useful in overcoming such transport problems by delivering the drug into the tumor-cell plasma membrane or beyond. Such considerations have been exploited successfully by Papahadjopoulos *et al.* (76) and Poste and Papahadjopoulos (77), who demonstrated that drug-resistance of transformed fibroblasts against actinomycin-D could be overcome by incubating the cells *in vitro* with liposome-associated actinomycin-D. Another example of the *in vitro* use of liposomes as carriers of antitumor drugs is the observation of Mayhew *et al.* (78) that liposome-entrapped 1-β-D-arabinofuranosyl-cytosine (Ara-C) is markedly more effective against L1210 cells than the free drug. Similar results have been reported from *in vivo* experiments, with Ara-C (44, 78,79) and actinomycin-D (80), as well as with other antitumor agents such as methotrexate (81) and 8-azaguanine (82). Chapter V contains some of our own observations on the *in vivo* efficacy of liposome-encapsulated Ara-C against a

mouse spleen tumor. One of the impediments to a full pharmacological use of liposomes as carriers in *e.g.* cancer chemotherapy, is their lack of "target"-specificity; as already indicated above, liposomes administered by intravenous injection, are for the greater part caught by the reticulo-endothelial system. The tissue distribution of liposomes can be altered quantitatively to some degree by varying their lipid compositions and size, and qualitatively by chosing different routes of administration (5,13,15,83). Nevertheless, the variability of such parameters seems to be limited. There have been some successful efforts to attain cell- or tissue-"targetting" using antibody-coated liposomes (84-89). Weinstein *et al.* (90) recently reported a promising type of *in vivo* "physical" targetting, which ultilizes local hyperthermia to induce local release of methotrexate from liposomes circulating in the blood-stream. Further development of systems for antibody-mediated targetting of liposomes will greatly depend on the progress in our knowledge of (tumor)immunology and on the advances in liposome technology. Another pharmacological applicability of liposomes might consist in their use as a sustained-drug-release system (44, *cf.* Chapter V).

We will conclude this Chapter with a short outline of the tumor model system which was used in most of the experiments described in this thesis.

Tumor cells

Zajdela ascites hepatoma cells have developed from rat solid hepatomas obtained by chronic oral administration of dimethylaminoazobenzene, as described in detail by Zajdela (91). Our main reasons for choosing this tumor as a model system to study some aspects of *in vitro* vesicle-tumor cell interaction were twofold: Firstly, the cells are easy to grow and to handle, and secondly, they might be compared to their "progenitor" cells, rat hepatocytes, with respect to mode and extent of interaction with liposomes (92, *cf.* Chapter IV). Zajdela cells often appear as "mini"-clones of up to 30 firmly cohering cells which are believed to have developed from one single tumor cell by successive mitoses (91). Transmission electron-microscopy (Fig. 3) reveals that the ultrastructure of Zajdela cells differs considerably from that of hepatocytes. As compared to the latter, Zajdela cells (Fig. 3A) possess an irregularly shaped, extended-chomatin type nucleus. Microvilli are unevenly distributed along the surface. The mitochondria are aberrant and cytoplasmic glycogen is absent (Fig. 3B). At present we do not know whether the intercellular junctions (Figs. 3A and 3C) also comprise gap junctions. The ultrastructural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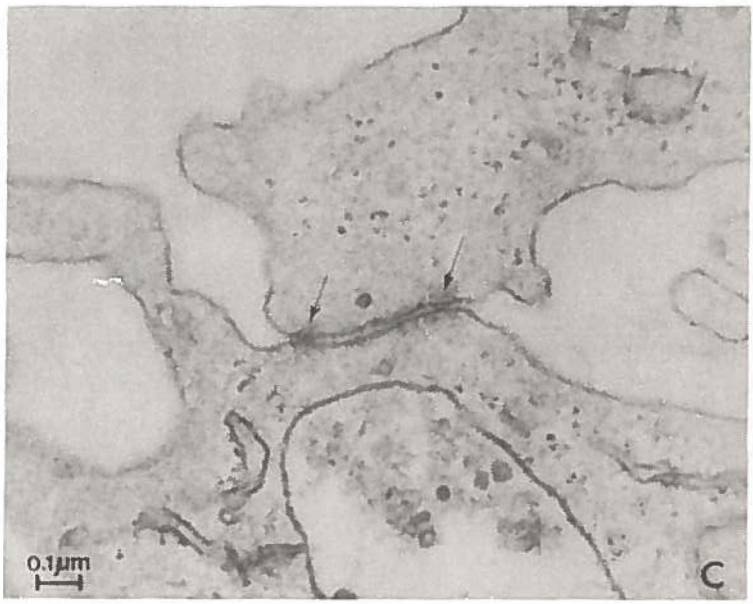

Fig. 3. *Ultrastructure of Zajdela ascites hepatoma cells.* The cells were isolated as described in Chapter II, fixed in 2% glutaraldehyde and post-fixed in 1% OsO_4, 1.5 % $K_4Fe(CN)_6$.

A. Two adhering cells; the arrows indicate intercellular junctions.

B. Detail of a cytoplasmic region; the cristae of the mitochondria are often dilated and irregularly arrayed. The rough endoplasmic reticulum is found exclusively in the form of single lamellae. m = mitochondrion; rer = rough endoplasmic reticulum.

C. High magnification of an intercellular junction. The arrows indicate tight junctions. (Courtesy of Dr. C.E. Hulstaert)

appearance of the Zajdela cells marks them as being highly anaplastic (*cf.* Ref. 92).

In contrast to *e.g.* mouse leukemia L1210- or Ehrlich ascites cells, Zajdela cells are not widely used as a tumor model system. Yet a number of their structural and functional characteristics have been studied, often in comparison to normal rat hepatocytes. These studies focussed on: membrane phospholipid composition (94), cell-surface binding-sites for various lectins (95-99), cell-surface receptors for adrenalin (100), glucocorticoids (101) and insulin (102), plasma membrane antigens (103), the mitochondrial adenosine triphosphatase (104-106), effect of calcium ions on mitochondrial respiratory function (107), uptake and processing of amino acids, nucleic acids, and precursors of nucleic acids (108,109), synthesis and fate of α-fetoprotein, fibrinogen and albumin (110,111), transport of proteins between cytoplasm and nucleolus (112), protein pattern of cytoplasmic ribosomes (113), DNA-dependent RNA-polymerase (114), characterization of the nuclear matrix (115), and the DNA-polymerase (116).

For our purposes, cells were implanted weekly by injection of 0.5 - 1.0 ml of ascitic fluid, containing approximately 10^7 cells, into the peritoneal cavity of ether-anesthetized adult female Wistar rats. Generally, the cells were harvested on the fifth day after implantation, by aspiration of 15-20 ml of sanguinolent ascitic fluid, containing (on the average) 3×10^8 cells. The presence of cells within the peritoneal cavity causes gross inflammatory reactions and intraperitoneal extravasation. If the tumor is given a full chance to develop, the animals die in severe cachexia 8-9 days after implantation.

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CHAPTER II

IN VITRO INTERACTION OF ZAJDELA ASCITES HEPATOMA CELLS WITH LIPID VESICLES

SUMMARY

We studied the *in vitro* interaction between Zajdela ascites hepatoma cells and small unilamellar vesicles, consisting of [^{14}C]labeled phosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio 5:4:1), containing high intravesicular concentrations of carboxyfluorescein or fluorescein isothiocyanate tagged dextran.

The entrapped markers were found to be associated with the cells to a lesser degree than the vesicle membrane marker. This discrepancy, which is slightly less pronounced for fluorescein isothiocyanate tagged dextran than for carboxyfluorescein, increases with incubation time and decreases with increasing vesicle lipid concentration in the incubation mixture. Vesicle-plasma membrane exchange of the vesicle lipid marker could not entirely explain the observed discrepancy. It is tentatively concluded that the gap mainly arises from a selective loss of entrapped dyes from vesicles actually interacting with the cell surface. Both spectrofluorimetric and fluorescence microscopic observations, as well as the relative insensitivity of vesicle uptake towards the presence of metabolic inhibitors, exclude a major contribution of endocytosis as a vesicle uptake route. We therefore conclude that vesicles are primarily internalized by a vesicle-cell fusion-like process. The observed discrepancy in uptake between entrapped materials and vesicle lipid is discussed in terms of a two-site vesicle-cell surface interaction model.

MATERIALS

Phosphatidylcholine and phosphatidylserine were isolated from total egg yolk lipids and from bovine brain extract (Sigma), respectively, by preparative thin layer chromatography. Cholesterol (CH-S grade), cholesteryl oleate, sodium azide, 2-deoxyglucose, and HEPES were purchased from Sigma. RPMI 1640

tissue culture medium was from Flow Labs. Inc. All lipids used gave one spot upon thin layer chromatography.

[1α,2α(n)-^{3}H] cholesterol (43 mCi/μmol), cholesteryl[1-^{14}C] oleate (15.4 μCi/μmol), and [6,6'(n)-^{3}H] sucrose (1Ci/mmol) were from The Radiochemical Center (Amersham, England). [*Me*-^{14}C] phosphatidylcholine was prepared from egg yolk phosphatidylcholine according to Stoffel *et al.* (1). Its specific activity varied between 2 and 3 μCi/μmol. Carboxyfluorescein was bought from Eastman Kodak Co. (Rochester, N.Y.), and purified as described by Blumenthal *et al.* (2). 100 mM stock solutions of carboxyfluorescein in H_2O, adjusted to pH 7.4 with 1 M NaOH, were kept in the dark until use. FITC-dextran was obtained from Pharmacia. This compound had an average molecular weight of 3000, and carried 1 FITC residue per 100 glucose units. For entrapment in vesicles 100 mM FITC-dextran solutions in NaCl/Hepes buffer were used.

METHODS

Cells

Zajdela ascites hepatoma cells (in the following referred to as Zajdela cells) were a generous gift from Dr. F. Zajdela, I.N.S.E.R.M., Paris. The cells, derived from a dimethylaminoazobenzene-induced hepatoma (3), were grown in the peritoneal cavity of adult female Wistar rats, and passed weekly to fresh animals. Cells were harvested by aspiration of ascitic fluid 5 days after implantation, in yields of approximately 3 x 10^{8} cells per animal, and subsequently freed of contaminating non-tumor cells by repeated centrifugations and washings in 0.135 M NaCl/0.010 M HEPES, brought to pH 7.4 with 1 M NaOH (this buffer will be referred to as NaCl/Hepes buffer), Cell counts were performed in a Bürker hemocytometer. Throughout the experiments more than 95% of the cells excluded Trypan Blue, indicating preserved viability.

Liposomes

Chloroform solutions of phosphatidylcholine, cholesterol, and phosphatidylserine were mixed in a molar ratio of 5:4:1, and taken to dryness, under reduced pressure, in a rotatory evaporator. The dried lipid film was vortex-dispersed either in NaCl/Hepes buffer for preparation of empty vesicles or in 100 mM aqueous solutions of carboxyfluorescein or FITC-dextran for preparation of loaded vesicles. Generally the final total lipid concentration in the dispersions was 8.75 mM. The lipid dispersions were exhaustively sonicated at

room temperature under a N_2-atmosphere, either during 1 hour intermittently (50% of the duty cycle) with a Branson B-15 sonifier equipped with a titanium microprobe, or during at least two hours in a Bransonic 220 bath sonicator. Both methods, as judged by Sepharose 4B chromatography, gave similar vesicle populations with respect to average size, with more than 95% of the lipid present as small unilamellar vesicles. Fluorophore containing vesicles were then freed from non-entrapped dye by chromatography on Sephadex G-100 (for carboxyfluorescein), or Sepharose 6B (for FITC-dextran). Columns (25 x 1 cm) were equilibrated and eluted with NaCl/Hepes buffer.

Incubations

Unless stated otherwise (see legends of Figs. and Tables), 5×10^7 - 2.5×10^8 Zajdela cells were incubated for 1 hour at 37 °C in a shaking water bath with 5 - 25 µmoles of vesicle lipid, in a final volume of 5 - 25 ml, in open Erlenmeyer flasks. The incubation medium consisted of a 1 : 5 (v/v) mixture of NaCl/Hepes buffer and RPMI 1640 medium, the latter supplemented with 25 mM HEPES and 10 mM $NaHCO_3$. The medium was brought to pH 7.4 with 1 M NaOH. No serum was added. At suitable times, duplicate 1-ml samples (containing 10^7 cells and 1 µmole of vesicle lipid) were withdrawn from the incubation mixture, transferred to polystyrene tubes, centrifuged (600 x g, 30 s) and washed twice with NaCl/Hepes buffer at room temperature. The samples were then taken to fresh polystyrene tubes, centrifuged and washed again, and finally suspended in 3 ml NaCl/Hepes buffer. For determination of cell-associated radioactivity 1 ml aliquots of the washed cell suspensions were pipetted into glass vials, mixed with 5 ml of xylene-based scintillation mixture (4) and counted in a Nuclear Chicago MKII liquid scintillation counter. The remainder of the 3 ml cell suspensions was used for fluorescence measurements with a Perkin Elmer MPF43 fluorescence spectrophotometer. Excitation and emission were at 490 and 520 nm, respectively. The instrument was calibrated with a 10^{-4} M solution of quinine sulfate in 0.05 M H_2SO_4. Cell-associated fluorescence was read in a 1 cm light path quartz fluorescence cuvette against series of freshly prepared carboxyfluorescein- or FITC-dextran standards in NaCl/Hepes buffer. Details on the processing of fluorescence measurements are described in the Appendix. Fluorescence microscopy was performed with a Leitz Orthoplan fluorescence microscope, equipped with a filter combination for fluorescein.

INTRODUCTION

In the past few years a vast number of investigations on the interaction between liposomes[a] and cells has been published (for reviews see 5,6). Since many investigators have indicated a possible role for liposomes as carriers of antitumor drugs in the chemotherapeutic treatment of cancer, we were interested in the details of the interaction between liposomes and the cells of an experimental animal tumor model system. For this study the highly anaplastic Zajdela ascites hepatoma (7) was chosen. Our first aim was to find an answer to some crucial questions: are Zajdela ascites hepatoma cells capable of taking up liposomes? Does this uptake[b], if occurring, consist of mere adsorption of intact vesicles to the cell surface, or are vesicles internalized within the cells? Furthermore: if internalization can be demonstrated, what is the mechanism of uptake? In 1977, developing an idea of W.A. Hagins, Weinstein *et al.* (8) introduced an elegant and sensitive method to quantitate vesicle-cell interaction. This method (for details see 2,8,9) utilizes high, self-quenching concentrations of a water-soluble fluorophore, carboxyfluorescein, trapped in the inner aqueous compartment(s) of lipid vesicles. The primary interaction between cell and vesicle will involve the attachment of the vesicle membrane surrounding the vesicle inner aqueous compartment(s) to the cell. If this is to be followed by vesicle-cell fusion, the subsequent transfer of the inner aqueous compartment(s) of the vesicle into the cytoplasm will result in a vast dilution of the entrapped concentrated dye into the cellular cytoplasm, causing complete relief of self-quenching and giving rise to an immediate fluorescent signal from the interior of the cell. If, on the other hand, the vesicles are predominantly endocytosed, the development of a fluorescent signal will depend mainly on the rate of leakage of the concentrated fluorophore from endocytotic vacuoles and/or secondary

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a) In this report the terms "liposomes" and "vesicles" will be used interchangeably.

b) The use of the term "uptake" does not imply internalization of vesicles by cells, but it is used as short for "association with cells". Whenever internalization is meant, this will be explicitely stated. If, in the text, terms like "total cell-associated carboxyfluorescein" are followed by a symbol, *e.g.* (Π_i), the reader is referred to the Appendix for its meaning. The term "adsorbed carboxyfluorescein" is to be read as short for: carboxyfluorescein, whose self-quenching is relieved upon addition of detergent.

lysosomes into the cytoplasm. Addition of a detergent (*e.g.* Triton X-100) to cells and vesicles will instantaneously set free any entrapped, self-quenched dye and thus allow determination of the fraction of carboxyfluorescein, which is still contained in intact vesicles, either absorbed to the plasma membrane or present in endocytotic vacuoles and/or secondary lysosomes.

In this report small unilamellar vesicles consisting of phosphatidylcholine, cholesterol, and phosphatidylserine in a molar ratio of 5:4:1 were used for several reasons. The choice of the lipid composition was based on the fact, that phosphatidylcholine/phosphatidylserine (molar ratio 9:1) vesicles have been suggested successful in transferring entrapped materials to a number of different cells, most likely via a vesicle-cell fusion mechanism (10). Incorporation of at least 40 mole % cholesterol into the vesicle membrane was required (see Results) to keep carboxyfluorescein leakage rates at acceptable levels during 1 hour incubations. We preferred the use of small unilamellar vesicles to the use of multilamellar vesicles because, in case of vesicle-cell fusion, all vesicle-entrapped fluorophore would be diluted at once, either into the cytoplasm and/or into the surrounding medium ("leaky fusion"). Furthermore, small unilamellar vesicles are both physically better defined and geometrically more homogeneous than multilamellar vesicles (11). In our experiments the uptake of vesicle-entrapped carboxyfluorescein was compared directly to uptake of vesicle-entrapped FITC-dextran, another highly water-soluble fluorescent compound with an average molecular weight of about 8 times that of carboxyfluorescein and having fluorescent spectral properties nearly identical to those of carboxyfluorescein. This report describes the results of a series of incubations of cells with vesicles, in which special attention was paid to the concordance between uptake of vesicle lipid and uptake of either entrapped dye in the same experiment.

RESULTS

Introductory experiments showed us that incorporation of 40 mole % of cholesterol in phosphatidylcholine/phosphatidylserine mixtures resulted in a three-fold decrease of vesicle permeability towards carboxyfluorescein. All experiments in this study were therefore done with vesicles composed of phosphatidylcholine, cholesterol and phosphatidylserine in a 5:4:1 molar ratio.

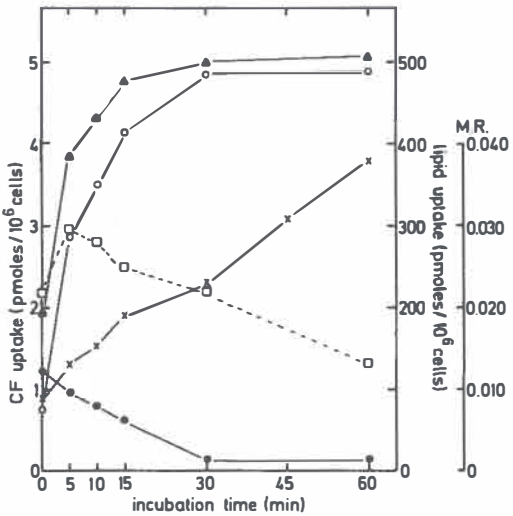

Fig. 1. *Uptake of vesicle-entrapped carboxyfluorescein and of total vesicle lipid as a function of time.* 1.75 x 10^8 Zajdela cells were incubated (37 °C, shaking water bath) with 17.5 µmoles of total vesicle lipid in a final volume of 17.5 ml in an open Erlenmeyer flask. Vesicles were composed of PC/Chol/PS (molar ratio 5:4:1) with 5 µCi of [*Me*-^{14}C]PC (specific activity 2.2 µCi/µmol) as a marker, and contained 100 mM CF. At suitable times duplicate 1 ml aliquots were withdrawn from the incubation mixture and processed as described in the Methods section and Appendix. X——X: uptake of total vesicle lipid based on uptake of [*Me*-^{14}C]PC. ▲——▲: total cell-associated CF (Π_i: see Appendix). O——O: CF inside cells (Π_d; see Appendix). ●——●: "adsorbed" CF (Π_a: see Appendix). Each point represents the average of duplicate measurements, that agree within 5%. The accessory ordinate at the extreme right of the Fig. designates the molar ratio (M.R.) of total cell-associated CF to total cell-associated vesicle lipid. Values for M.R. were obtained by dividing the number of pmoles of total CF associated with 10^6 cells by the number of pmoles of vesicle lipid associated with 10^6 cells. From fluorescence readings after addition of detergent and radioactivity determinations the molar ratio of total entrapped CF to total vesicle lipid in the original vesicle preparation was calculated: 0.030. □——□ : Molar ratio (M.R.) of total cell-associated CF (Π_i) to total cell associated vesicle lipid. Abbreviations: PC: phosphatidylcholine; PS: phosphatidylserine; Chol: cholesterol; CF: carboxyfluorescein.

Fig. 1 shows simultaneously measured uptake of vesicle lipid and vesicle-entrapped carboxyfluorescein as a function of incubation time. Both the amount of total cell-associated dye (Π_i), and the amount of dye inside the cells (Π_d) reach maximum values after about 30 min. The fraction of carboxy-

fluorescein which can be measured only after addition of detergent (Π_a) decreases from a maximum value at zero-time to a constant low value at longer incubation times. The uptake of vesicle lipid by the cells, based on [^{14}C] phosphatidylcholine, increases steadily with time, during the first hour of incubation. All uptake curves show relatively high zero-time[a] values, indicating that vesicle-cell contact occurs immediately upon addition of vesicles to cells. The molar ratio of total cell-associated carboxyfluorescein to total cell-associated vesicle lipid has a maximum value after 5 min of incubation and steadily decreases with time, suggesting a progressive loss of dye from vesicles and/or cells into the incubation medium. Since the carboxyfluorescein uptake values shown were corrected for leakage of dye from the bulk of vesicles in the incubation mixture (see Appendix), such a loss of carboxyfluorescein could be considered to result from the contact between vesicles and cells. The magnitude of this phenomenon, as well as the absolute uptake values of carboxyfluorescein and vesicle lipid were found to vary within ± 50% with the cell preparation used. The time-dependent decrease of the dye: lipid ratio, however, was observed in all experiments, and the shapes of dye- and lipid uptake curves were also consistently similar throughout the experiments. The possibility of a time-dependent decrease in carboxyfluorescein quantum yield as a result of metabolic modifications of the molecule was exluded by the observation that there was no change in quantum yield of the dye during a 3 h incubation at 37 °C in concentrated cell lysates.

Detailed examination (not shown) of the time interval between 0 and 15 min revealed that uptake of both carboxyfluorescein (Π_i) and of vesicle lipid is linear up to 5 min. We therefore measured uptake of dye and lipid as a function of vesicle lipid concentration after 5 min of incubation as is shown in Fig. 2. Both total cell-associated carboxyfluorescein (Π_i), as well as the actually internalized fraction of carboxyfluorescein (Π_d), gradually increase with increasing vesicle lipid concentration. By contrast, the "adsorbed" dye fraction (Π_a,

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a) It should be kept in mind, that the processing of samples from the incubation mixture takes approximately 3 min, so that "zero-time" in our experiments is not identical to physical zero-time. In this report, zero-time refers to sampling.

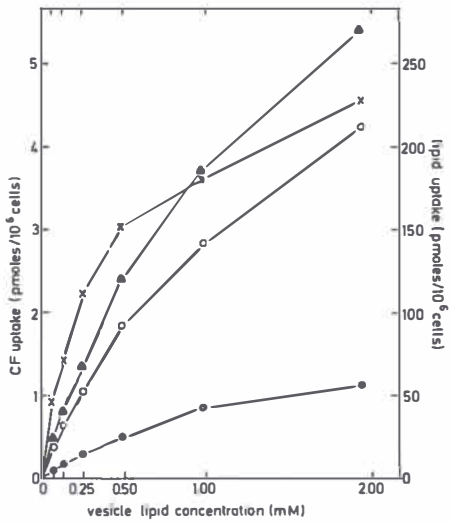

Fig. 2. *Uptake of vesicle-entrapped carboxyfluorescein and of total vesicle lipid as a function of the total vesicle lipid concentration in the incubation mixture.* For each vesicle lipid concentration studied, 5 x 10^7 Zajdela cells were incubated (37 °C, shaking water bath) with suitable dilutions of one vesicle preparation, composed of PC/Chol/PS (molar ratio 5:4:1) with 10 µCi of [*Me*-^{14}C]PC (2.2 µCi/µmol) as a marker, in a final volume of 5 ml, in open Erlenmeyer flasks. After 5 min of incubation, duplicate 1 ml samples were withdrawn from each vessel and processed as described in the Methods section and Appendix. X——X: uptake of total vesicle lipid based on the uptake of [*Me*-^{14}C]PC. ▲——▲: total cell-associated CF (Π_i: see Appendix). O——O: CF inside cells (Π_d; see Appendix). ●——●: "adsorbed" CF (Π_a; see Appendix). Each point represents the average of duplicate measurements that agree within 5%. For abbreviations see the legend of Fig. 1.

see Appendix), saturates at high vesicle lipid concentrations. From a double reciprocal plot of "adsorbed" carboxyfluorescein *vs.* vesicle lipid concentration we calculated that half of the maximum value of 1.5 pmoles of "adsorbed" dye per 10^6 cells is reached at a vesicle lipid concentration of 0.82 mM. From Fig. 2 it is clear that the molar ratio of total cell-associated carboxyfluorescein (triangles) to total cell-associated lipid (crosses) increases with vesicle lipid concentration. The actual values of these ratios are presented in Table 1, to be compared with the corresponding ratios in the entire incubation mixture, which are fairly constant. Only at high vesicle lipid

Table 1

MOLAR RATIOS OF CARBOXYFLUORESCEIN : LIPID IN THE INCUBATION MIXTURES *VS.* IN THE CELLS

Data in this table are taken from the experiment described in Fig. 2.
Middle column: Values were obtained as follows: to duplicate 50 µl samples from each incubation vessel Triton X-100 was added to a final concentration of 1% (v/v); fluorescence was read and radioactivity determined. The subsequently calculated number of pmoles CF was divided by the calculated number of pmoles of total vesicle lipid, per unit volume, yielding the initial molar ratio*.
Right column: Values were obtained from the values shown in Fig. 2 as follows: the number of pmoles of total cell-associated CF was divided by the number of pmoles of total cell-associated vesicle lipid, for each incubation vessel. Thus the molar ratio** of cell-associated markers is obtained.
For experimental conditions and statistics see the legend of Fig. 2. The molar ratio of entrapped CF to total vesicle lipid in the original vesicle preparation was 0.027. For abbreviations see the legend of Fig. 1.

Vesicle lipid concentration (mM)	Molar ratio* CF/lipid	Molar ratio** CF/lipid
0.059	0.029	0.010
0.119	0.029	0.011
0.238	0.026	0.012
0.475	0.027	0.016
0.950	0.027	0.021
1.900	0.027	0.023

* molar ratio of entrapped CF to total vesicle lipid in each incubation vessel.
** molar ratio of total cell-associated CF to total cell-associated lipid.

concentration there is good agreement between the bulk ratio and the cell-associated ratio. Apparently, the selective loss of dye, probably resulting, as discussed above, from vesicle-cell contact, decreases with increasing vesicle lipid concentration. With FITC-dextran, to be described two paragraphs below as entrapped fluorophore, a similar phenomenon is observed.

When attempting to explain the observed changes in molar ratios three

possibilities should be considered. First, vesicles plus contents are internalized, but contents (carboxyfluorescein) leak from the cells rapidly. Determination of leakage rates (average half-life of 14 min) of the dye from cells preloaded with free dye indicated that such a process may indeed contribute. Second, carboxyfluorescein leaks preferentially from vesicles attached to the cell surface. The observation that "adsorbed" dye (Fig. 1, filled circles) rapidly decreases to a very low value is in line with such a view, as will be outlined in the Discussion. Third, uptake of [^{14}C] phosphatidylcholine is not representative of uptake of whole vesicles but, in part, reflects exchange of lipid between vesicle and cell membrane (5).

Since carboxyfluorescein leaks out of vesicles and cells fairly rapidly we set out to test a compound with similar fluorescence properties but higher molecular weight: FITC-dextran. In contrast to carboxyfluorescein, FITC-dextran at an intravesicular concentration of 100 mM, which is about the highest FITC-dextran concentration still allowing formation of vesicles, is only about 65% self-quenched. Hence, amounts of internalized or "adsorbed" FITC-dextran cannot be calculated as accurately as was done for carboxyfluorescein, which is nearly 100% self-quenched in such conditions (also see Appendix). Yet, FITC-dextran has two major advantages over carboxyfluorescein: the overall leakage-rate of the dextran from vesicles in an incubation with cells is less than half that of carboxyfluorescein, and, in addition, FITC-dextran leakage is barely dependent on the environmental pH, in contrast to carboxyfluorescein, which at pH 5.8 leaks from vesicles about 3.5 times as fast as at pH 7.4.

Fig. 3 shows that, during incubation of cells with free FITC-dextran, cell-associated FITC-dextran fluorescence, as measured before addition of detergent, reaches a nearly constant level in about 10 min. In contrast, total cell-associated FITC-dextran fluorescence, as measured after addition of detergent, increases during up to 60 min of incubation time. Since, at the concentration added, FITC-dextran shows no significant self-quenching, the discrepancy between fluorescence readings before and after addition of detergent must be due to accumulation of the dye to high intracellular concentrations or/and into subcellular compartment(s) of low average pH. Either possibility indicates, that most likely the free compound is endocytosed and ultimately accumulates in the lysosomes. This interpretation is supported by the

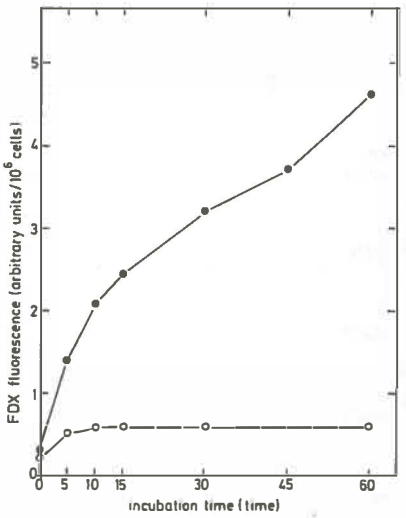

Fig. 3. *Uptake of free FITC-dextran in the presence of empty vesicles as a function of time.* 1.75 x 10^8 Zajdela cells were incubated (37 °C, shaking water bath) with 17.5 µmoles of total vesicle lipid plus 45 µM (final concentration) free FDX in a final volume of 17.5 ml in an open Erlenmeyer flask. Empty vesicles consisting of PC/Chol/PS (molar ratio 5:4:1) had been previously mixed with free FDX. At suitable times duplicate 1 ml samples were taken from the incubation mixture and processed as described in the Methods section and Appendix. ●——●: cell-associated FDX fluorescence, as measured after addition of detergent. O——O: cell-associated FDX fluorescence as measured before addition of detergent. Each point represents the average of duplicate measurements that agree within 5%. Filled circles: 1.5 arbitrary units/10^6 cells correspond to 1 pmole of FDX/10^6 cells. Abbreviations: FDX stands for FITC-dextran; also see legend of Fig. 1.

observation that during prolonged incubation, after removal of free FITC-dextran, total cell-associated fluorescence remains constant, whereas fluorescence, as measured before addition of detergent, slowly decreases with time. We consider it unlikely that the dye is concentrated within secondary lysosomes to an extent which would give rise to significant self-quenching: even at high intralysosomal FITC-dextran concentrations a marked residual fluorescence (Λ_o, see Appendix) would remain. Therefore the ratio of fluorescence values between upper and lower curve in Fig. 3 can be considered as a measure for the fraction of FITC-dextran molecules that is exposed to low (intralysosomal) pH.

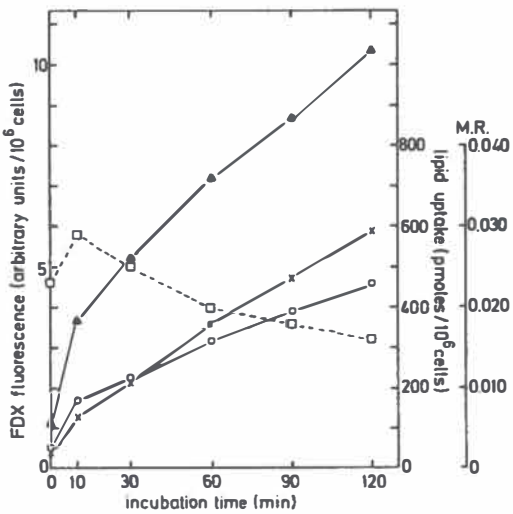

Fig. 4. *Uptake of vesicle-entrapped FITC-dextran and of total vesicle lipid as a function of time.* 1.4 x 10^8 Zajdela cells were incubated (37 °C, shaking water oath) with 14 µmoles of total vesicle lipid in a final volume of 14 ml in an open Erlenmeyer flask. Vesicles were composed of PC/Chol/PS (molar ratio 5:4:1) with 5 µCi of [*Me*-^{14}C]PC (specific activity 2.2 µCi/µmole) as a marker, and contained 100 mM FDX. At suitable times duplicate 1 ml samples were taken from the incubation mixture and processed as described in the Methods section and Appendix. X——X: uptake of total vesicle lipid based on the uptake of [*Me*-^{14}C]PC. ▲——▲: cell-associated FDX fluorescence, as measured after addition of detergent. O——O: cell-associated FDX fluorescence, as measured before addition of detergent.
Each point represents the average of duplicate measurements, that agree within 5%. The accessory ordinate at the extreme right of the Fig. designates the molar ratio (M.R.) of total cell-associated FDX to total cell-associated vesicle lipid. Values for M.R. were obtained by dividing the number of pmoles of FDX associated with 10^6 cells by the number of pmoles of vesicle lipid associated with 10^6 cells. From fluorescence readings after addition of detergent and radioactivity determinations the molar ratio of total entrapped FDX to total vesicle lipid in the original vesicle preparation was calculated: 0.034. □——□: molar ratio (M.R.) of total cell-associated FDX to total cell-associated vesicle lipid. Filled triangles: 10 arbitrary units/10^6 cells correspond to 9.34 pmoles of FDX/10^6 cells. For abbreviations see the legends of Figs. 1 and 3.

In this view it is interesting to see the results of others, who used high molecular weight FITC-dextran for the measurement of the intralysosomal pH (12).

When, by contrast, vesicle-entrapped FITC-dextran is offered to the cells, cell-associated FITC-dextran fluorescence, as measured before addition of detergent, steadily increases with time (Fig. 4). Total cell-associated FITC-dextran fluorescence, as measured after addition of detergent, matches the uptake of vesicle lipid slightly better than in case we used vesicle-entrapped carboxyfluorescein (Fig. 1). Yet, the molar ratio of dye to lipid still tends to decrease after reaching a maximum at short incubation time. The difference in FITC-dextran uptake characteristics between Figs. 3 and 4 suggests, that vesicle-entrapped FITC-dextran is, at least partly, delivered directly into the cytoplasm.

In order to test to what degree cells are able to retain vesicle lipid and vesicle-entrapped carboxyfluorescein or FITC-dextran, we incubated cells for 45 min with vesicles containing either type of dye, removed non cell-associated vesicles, and continued incubation for another 60 min in vesicle-free, fresh medium. The results are shown in Fig. 5. Lipid from both types of vesicles remains firmly cell-associated, indicating that neither intact vesicles nor water-soluble metabolites of the lipid marker are released into the medium. Vesicle-transferred carboxyfluorescein leaks from the cells almost completely, with a half-life of approximately 15 min. By contrast, vesicle-transferred FITC-dextran is released only to a limited extent: about 65% of the vesicle-transferred molecules remain associated with the cells. These findings are in line with what we observed in Figs. 1 and 4: a higher degree of concordance between uptake of FITC-dextran and lipid (Fig. 4) than between carboxyfluorescein and lipid (Fig. 1). Cell-associated carboxyfluorescein, as measured before addition of detergent, follows the same decay pattern as total cell-associated carboxyfluorescein. This means that, at any time, all cell-associated carboxyfluorescein molecules show the same change in quantum yield upon addition of detergent. Thus, there is no unidirectional transport of the dye between subcellular compartments during the continued incubation. Most likely, carboxyfluorescein readily diffuses out of the cytoplasm. With FITC-dextran, only a 10% decrease in fluorescence can be measured directly (*i.e.* before addition of detergent) during continued incubation, whereas a

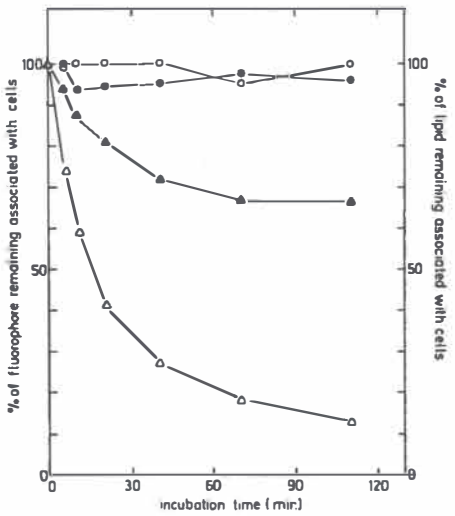

Fig. 5. *Release of vesicle lipid and vesicle-transferred fluorophore (FITC-dextran or carboxyfluorescein) from cells as a function of time.* 2.5 x 10^8 Zajdela cells were incubated in a final volume of 25 ml with 25 moles of total vesicle lipid containing either 100 mM CF or 100 mM FDX. The vesicles were composed of PC/Chol/PS (molar ratio 5:4:1), and carried 5 µCi of [*Me*-^{14}C]PC (specific activity 3 µCi/µmol) as a lipid marker. Incubations were carried out at 37 °C (shaking water bath) in open Erlenmeyer flasks. After 45 min of incubation the cells from each incubation vessel were centrifuged (30 sec, 600 x g) and washed twice to remove non cell-associated vesicles. Subsequently, 25 ml of fresh, vesicle-free medium were added to the cells in each vessel, and the incubation was continued. At suitable times duplicate 1 ml aliquots were taken from the incubation mixtures and processed as described in the Methods section and Appendix. Lipid- and fluorophore amounts remaining associated with the cells are expressed as percent of values determined immediately after medium-change (zero-time). ▲——▲: total cell-associated FDX. △——△: total cell-associated CF. ●——●: total cell-associated vesicle lipid from vesicles containing 100 mM FDX. O——O: total cell-associated vesicle lipid from vesicles containing 100 mM CF.
Each point represents the average of duplicate measurements that agree within 5%. For abbreviations see the legends of Figs. 1 and 3.

35% decrease is found after addition of detergent. This suggests that the greater part of FITC-dextran loss from the cells does not occur from within the cells, but rather from vesicles attached to the cell surface.

In view of the observed incubation-time dependent discrepancies between lipid-uptake and fluorophore-uptake we included two additional labels in our

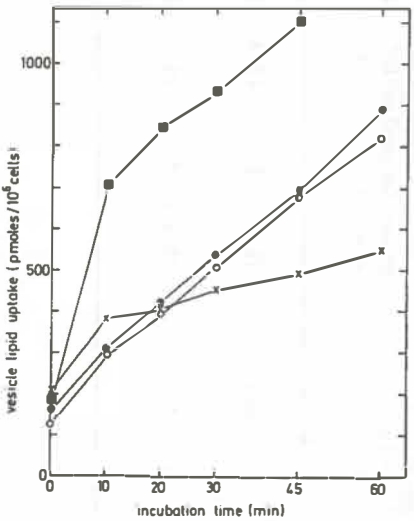

Fig. 6. *Uptake of empty vesicles, based on 3 different lipid radioactive markers, as a function of time.* For each type of vesicle preparation 1.875 x 10^6 Zajdela cells were incubated with 15 µmoles of total vesicle lipid in a final volume of 15 ml. The incubations were carried out in open Erlenmeyer flasks at 37 °C (shaking water bath). At suitable times duplicate 1 ml samples were taken from the incubation mixtures and processed as described in the Methods section. ■——■: PC/Chol/PS (molar ratio 5:4:1) with 5 µCi of [1α, 2α (n)-^{3}H]cholesterol (specific activity 43 mCi/µmol) as a marker. ●——●: PC/Chol/Chol.ol./PS (molar ratio 5:3.87:0.13:1) with 3 µCi of [*Me*-^{14}C]PC (specific activity 3 µCi/µmol) as a marker. O——O: PC/Chol/PS (molar ratio 5:4:1) with 3 µCi of [*Me*-^{14}C]PC (specific activity 3 µCi/µmol) as a marker. X——X: PC/Chol/Chol.ol/PS (molar ratio 5:3.87:0.13:1) with 3 µCi of cholesteryl [1-^{14}C]oleate (15.4 µCi/µmol) as a marker.
Each point represents the average of duplicate measurements that agree within 5%. Abbreviations: Chol.ol. stands for cholesteryl oleate; also see the legend of Fig. 1.

experiments: cholesterol and cholesteryl oleate, the former known to exchange readily between membranes (13), the latter being considered as non-exchangeable lipid. Empty vesicles, made of phosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio 5:4:1), with or without a trace of cholesteryl oleate, were incubated with the Zajdela cells. The vesicles were labeled in the phosphatidylcholine, cholesterol, or cholesteryl oleate. Fig. 6 shows that the incorporation of 2.3 mole % of cholesteryl oleate did not significantly affect uptake of phosphatidylcholine-labeled vesicles (open and filled

Table 2

INFLUENCE OF METABOLIC INHIBITORS ON THE UPTAKE OF [^{3}H]SUCROSE, FREE FITC-DEXTRAN AND VESICLE-ENTRAPPED FITC-DEXTRAN

5 x 10^7 Zajdela cells were incubated (37 ^{o}C, shaking water bath) for 60 min in open Erlenmeyer flasks, in a final volume of 5 ml, with either 5 µCi of [6,6'(n)-^{3}H] sucrose (spec. act. 1 Ci/mmol), 1 mM empty PC/Chol/PS (molar ratio 5:4:1) vesicles plus 40 µM free FDX, or 1 mM PC/Chol/PS (molar ratio 5:4:1) vesicles containing 100 mM FDX. For the latter preparation the trapping efficiency was such, that upon liberation of the entrapped FDX, a final concentration of 40 µM free FDX would result. The incubations were carried out in the absence (controls) or presence of 50 mM 2-deoxyglucose plus 5 mM sodium azide, added to the cells 30 min before starting the incubation. After 60 min of incubation 1 ml duplicate samples were withdrawn from each incubation, and processed as described in the Methods section and Appendix. Values represent the averages of duplicate measurements that agree within 5%.
For abbreviations see the legends of Figs. 1 and 3.

Addition	[^{3}H] sucrose uptake in pmoles/10^6 cells	Free FDX uptake in pmoles/10^6 cells		Vesicle-entrapped FDX uptake in pmoles/10^6 cells*	
		expt. 1	expt. 2	expt. 1	expt. 2
none (control)	0.50 (100%)	6.4 (100%)	4.6 (100%)	14.3 (100%)	12.2 (100%)
2-deoxyglucose (50 mM) plus sodium azide (5 mM)	0.24 (48%)	2.2 (34%)	2.0 (43%)	13.9 (97%)	10.2 (84%)

* total cell-associated FDX

circles) by the cells. Transfer from both types of vesicles is linear for at least an hour and, when calculated as total vesicle lipid, 800 - 900 pmoles per 10^6 cells are taken up in this period. Based on [^{3}H]-labeled cholesterol, vesicle uptake values are more than twice as high as those based on [^{14}C]-phosphatidylcholine. With [^{14}C]-labeled cholesteryl oleate the initial values of total lipid uptake are somewhat higher than those based upon phosphatidylcholine, but during continued incubation the cholesteryl ester values are progressively lagging behind those of phosphatidylcholine. At present we have no explanation for the higher initial values. Possibly, cholesteryl esterase activity, which reportedly is associated with rat liver cytosol and lysosomes (14), is present in the plasma membranes, leading to some transfer of free radiolabeled oleate to the cells. We are currently investigating this possibility.

Table 2 shows to what extent uptake of free and vesicle-entrapped materials depends on metabolic energy supply: uptake of vesicle-entrapped FITC-dextran is only slightly affected by the presence of 2-deoxyglucose plus sodium azide, a combination known to effectively inhibit energy-dependent endocytosis. By contrast, uptake of free [^{3}H] sucrose and of free FITC-dextran is inhibited more than 50% when compared to the corresponding controls. These findings confirm that energy-dependent endocytosis does not play a significant role in the uptake of FITC-dextran containing vesicles.

Fluorescence microscopic observations on viable cells following incubation with either free or vesicle-entrapped fluorophore agree with the results presented so far: after incubation of cells with free carboxyfluorescein (Fig. 7.A), vesicle-entrapped carboxyfluorescein (Fig. 7.B), or vesicle-entrapped FITC-dextran (Fig. 7.D), an evenly distributed fluorescence is observed throughout the cytoplasm of most cells, the overall fluorescence intensity being highest in cells incubated with vesicle-entrapped carboxyfluorescein. Often the nuclei appear as relatively dark pits, indicating that fluorescence originates from within the cytoplasm. Occasionally, cells are found that show several brilliantly fluorescing spots. On morphological grounds (phase contrast microscopy) it was excluded that these cells are other than tumor cells. In case of vesicle-entrapped FITC-dextran (Fig. 7.D) very faint fluorescent rims can be seen at the cell periphery. These most likely are caused by vesicles adsorbed to the cell surface (FITC-dextran is not fully self-quenched inside vesicles, in contrast to carboxyfluorescein).

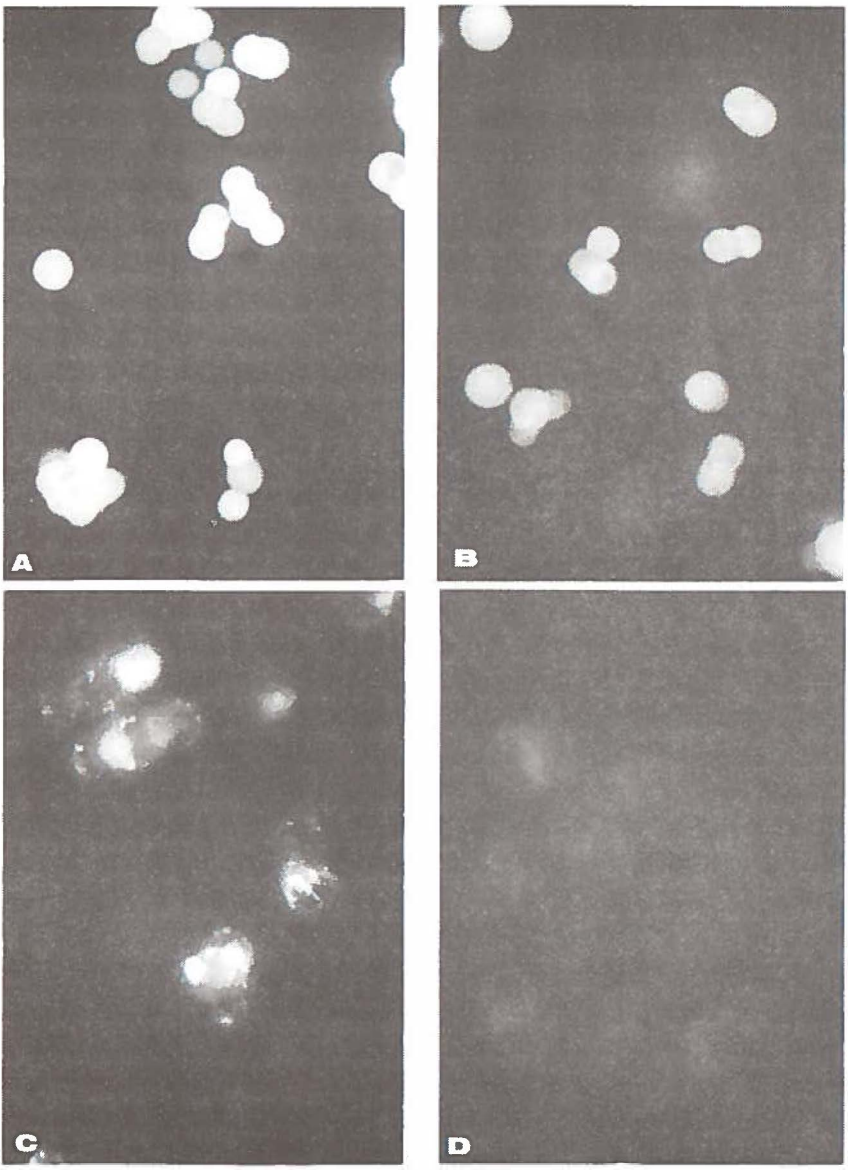

Fig. 7. *Fluorescence micrographs of Zajdela cells incubated with free- and vesicle-entrapped fluorophores.* Incubations(30 min) were carried out as described in the legends of Figs. 1, 3 and 4. Cells were incubated with (A) 1 mM empty vesicles plus 50 µM free CF (375 x); (B) 1 mM vesicles, containing 100 mM CF (375 x); (C) 1 mM empty vesicles plus 100 µM free FDX (600x); (D) 1 mM vesicles, containing 100 mM FDX (600 x). For abbreviations see the legends of Figs. 1 and 3.

Generally, fluorescence intensity is quite heterogenous throughout the cell population, with a small number of cells showing no fluorescence at all. When cells are incubated with free FITC-dextran (Fig. 7.C), most cells show a large number of brilliantly fluorescing spots. The presence of metabolic inhibitors does not cause a significant change in the appearance of cells upon incubation with either free- or vesicle-entrapped FITC-dextran.

These observations confirm our conclusion, that vesicle-entrapped fluorophores end up diffusely distributed in the cells, *i.e.* in the cytoplasm. For FITC-dextran this is in sharp contrast to the fate of the free dye which is intracellularly recovered in distinct compartments, most probably endocytotic vacuoles and/or lysosomes.

DISCUSSION

The basic conclusion from the work presented in this report, is that Zajdela cells are capable of internalizing the contents of small unilamellar vesicles, and that the entrapped volume is released directly into the cytoplasm. Evidence is obtained using both quantitative spectrofluorimetry and fluorescence microscopy. Generally, a discrepancy between uptake of entrapped markers and uptake of vesicle lipid is observed: during vesicle-cell incubation the entrapped markers are taken up to a lesser extent than the vesicle lipid. This discrepancy is slightly smaller when FITC-dextran is used instead of carboxyfluorescein as a vesicle-entrapped marker; it increases with the duration of vesicle-cell incubation, and decreases with increasing vesicle lipid concentration in the incubation mixture. With carboxyfluorescein as entrapped fluorophore the discrepancy is likely to be due to a combination of leakage of vesicle-derived carboxyfluorescein from the interior of the cell and from vesicles adhering to the cell surface. With FITC-dextran, on the other hand, the discrepancy is largely caused by leakage of the dye from vesicles attached to the cell surface. In both cases an overestimation of vesicle lipid uptake caused by vesicle-plasma membrane exchange of [^{14}C]-labeled phosphatidylcholine cannot be excluded. Arguments against a major involvement of this mechanism are: first, when using FITC-dextran as a vesicle-entrapped marker the discrepancy is less than with carboxyfluorescein as a marker (Figs. 1 and 4) and second, others (15,16) have shown phosphatidylcholine-exchange between vesicles and cell surface, when compared to total

vesicle lipid uptake, to be rather low at 37 °C. Also, the use of serum-free medium excludes a contribution of lipoprotein- or albumin-mediated phosphatidylcholine transfer between vesicles and cell surface (17).

The absolute levels of uptake of vesicle-entrapped marker and of total vesicle lipid are of the same order of magnitude as those obtained by others, working with other *in vitro* systems (10,18,19,20). Isolated hepatocytes, from which Zajdela cells are derived (3), however, seem to take up vesicles more efficiently (21). For short incubation times our data (Fig. 1) show, that not all vesicle-mediated carboxyfluorescein uptake could have occurred through an endocytotic route: the post-to pre-Triton fluorescence ratios (4.6,2.4,2.2 for 0,5,10 min of incubation time, respectively) are far too low as to indicate exclusive endocytosis of vesicles (in which case we would expect a ratio of 20-40). The fraction of the total vesicle population that is found associated with the cells at a given time, is very small: approximately 0.5% after 60 min of incubation. This, however, still represents a large number of vesicles associated with one single cell. If we assume one vesicle to be composed of 4000 lipid molecules (22) we can calculate that after 30 min of incubation some 35 000 vesicles will have become associated with one single cell. The experiments described offer circumstantial evidence that vesicles are primarily taken up by a vesicle-cell fusion-like mechanism. Arguments against a considerable involvement of endocytosis are found in the marked difference between uptake of free FITC-dextran and of vesicle-entrapped FITC-dextran (Figs. 3 and 4), in the relative insensitivity of vesicle-uptake to the presence of metabolic inhibitors (Table 2), and in the fluorescence microscopic images of cells following incubation with free FITC-dextran and vesicle-entrapped FITC-dextran. With carboxyfluorescein as an entrapped marker, estimation of a contribution of endocytosis is difficult: carboxyfluorescein has a rather low molecular weight, and its permeation through vesicle membranes was found to be considerably accelerated on lowering the environmental pH (see Results); hence a rapid passage of this dye through the lysosomal compartment cannot be excluded.

A major problem is envisaged during observation of time-dependent uptake of vesicle lipid and vesicle-entrapped markers: apparently the interaction process is initiated so fast that, despite rapid processing of incubation mixture samples, relatively high zero-time values are obtained for uptake of both vesicle lipid and entrapped fluorophores. This is important in view of

two observations: first, with either fluorophore the molar ratio of total cell-associated dye to total cell-associated vesicle lipid is consistently found to increase initially between 0 and 5 min of incubation time, and then to decrease gradually with longer incubation times (Figs. 1 and 4), and second, the fraction of carboxyfluorescein, which becomes unquenched upon addition of detergent, is maximal at zero time (Fig. 1). One explanation for these observations is the occurrence of rapid leakage of both fluorophores (when compared to their leakage from the bulk of vesicles in the incubation mixture) from vesicles that approach to or make actual physical contact with the cell surface at the onset of the interaction process. Once internalization of (other) vesicles has started to proceed, this "very leaky" fraction may become progressively masked. It is tempting to speculate on the existence of two major vesicle binding sites at the cell surface. Binding of a vesicle to one site would alter its structure such that entrapped markers would be released into the surrounding medium, whereas binding to the other site would result in an actual and complete capture of the vesicle lipid plus internalization of the vesicle contents. The results on concentration-dependent uptake of vesicle lipid and entrapped carboxyfluorescein (Fig. 2) lend support to this hypothesis: if the first binding site becomes nearly saturated, any further rise of the vesicle lipid concentration in the system is likely to result in a larger fraction of the interacting vesicles to be actually and completely internalized through the other binding site. Thus, the molar ratio of total cell-associated fluorophore to total cell-associated lipid would increase, as is observed. The amount of carboxyfluorescein, whose quenching is relieved upon addition of detergent (Π_a), reaches 75% of its maximal value at a vesicle lipid concentration of 1.9 mM (calculated from Fig. 2). By contrast, the amount of carboxyfluorescein, measurable before addition of detergent, does not simply saturate, as can be verified by constructing a double reciprocal plot. It would be of interest to see whether the site at which, in our hypothesis, complete capture of the vesicle lipid and -contents occurs, would be kinetically (and maybe physically) identical to the carboxyfluorescein transfer site, which was demonstrated to be present on the lymphocyte surface by Blumenthal *et al.* (2). At present it is not known whether liposome-mediated carboxyfluorescein-transfer is the result of complete vesicle-cell fusion involving integration of the vesicle lipid into the lipid backbone of the cellular plasma membrane, or that transfer of the dye occurs through an

alleviated permeability barrier consisting of two closely apposed bilayers (vesicle- and plasma membrane). Our results with FITC-dextran as an intravesicular marker clearly indicate that liposome-mediated carboxyfluorescein transfer is not a process intrinsically determined by the mere use of carboxyfluorescein as a vesicle-entrapped marker. To check the hypothesis outlined above, it would be important to gain a better insight in the kinetics of vesicle-cell interaction during the first few minutes of incubation. This, however, obviously requires a different experimental approach, involving instantaneous and complete separation of cells from bulk vesicles in an incubation.

An indication for the occurrence of a "very leaky" fraction of vesicles adsorbed to the cell surface, following vesicle-cell incubation, is provided by the experiment described in Fig. 5. Only a limited fraction of the total amount of FITC-dextran that becomes cell-associated upon incubation of FITC-dextran-containing vesicles with cells is released during subsequent continued incubation. At the same time, no significant loss of cell-associated vesicle lipid, either intact or metabolized, occurs. Free FITC-dextran, once taken up by the cells, remains firmly cell-associated, indicating that it is neither exocytosed nor released by diffusion across the plasma membrane. Hence it is very likely that the fraction of FITC-dextran leaving the vesicle-cell complex after preceding incubation of cells with FITC-dextran-containing vesicles, originates from vesicles adhering to the cell surface. It remains to be established, however, if and to what extent the size of this fraction depends on the duration of the preceding vesicle-cell incubation.

The observation that fluorescence intensity is quite heterogeneously distributed within the tumor cell population after incubation with dye-containing vesicles, might turn out to be of crucial importance in studying the anti-tumor drug carrier potential of liposomes. Laser flow microfluorimetric (23) investigations on synchronized tumor cell populations, following vesicle-cell incubations at different cell cycle stages, will provide useful information in this respect.

APPENDIX

The fluorescence reading obtained from the 2 ml of cell suspension remaining, after withdrawal of a 1 ml sample for radioactivity determination, is

called F_d (directly measurable fluorescence). After measuring F_d, 0.22 ml of a 10% (v/v) aqueous solution of Triton X-100 is added to the sample, followed by vortex mixing for several minutes. Then fluorescence is read again, and the value obtained is multiplied by 1.11, the dilution factor introduced by the addition of the detergent. This yields F_i (indirectly measurable fluorescence). In this way values for F_d and F_i are obtained from duplicate measurements, and the averages, $\bar{F}_d$ and $\bar{F}_i$, are calculated. The total amount of carboxyfluorescein or FITC-dextran associated with 10^6 cells and the amount of carboxyfluorescein inside 10^6 cells, Π_i and Π_d respectively, are then calculated in pmoles from the following equations:

$$\Pi_d = (\bar{F}_d - \bar{s}_d - \bar{f}_d)\ \mu\alpha k \qquad (1)$$

$$\Pi_i = (\bar{F}_i - \bar{s}_i - \bar{f}_i)\ \mu k \qquad (2)$$

s_d and s_i symbolize the autofluorescence plus light-scattering background from cells, treated in the same way as the samples from the incubation, but without exogenous fluorophore, f_d and f_i designate the fluorescence imparted to the cell sample by carboxyfluorescein, taken up as free dye after leakage from the vesicles during the incubation. If Zajdela cells are incubated with empty vesicles plus free carboxyfluorescein, the uptake of dye is linear with the concentration of free carboxyfluorescein in the incubation medium, for dye-concentrations up to 50 µM. From the leakage of carboxyfluorescein from the bulk of liposomes in the incubation medium (see below) the final concentration of free carboxyfluorescein in the incubation is known. Thus the amount of free dye taken up by the cells can be calculated, yielding f_d and f_i. For accurate determination of f_d and f_i, control incubations of cells with empty liposomes plus 25 µM free carboxyfluorescein were run in all experiments described. Generally, $f < 0.05\ F$. Range of s: $0.03\ F < s < 0.30\ F$ for carboxyfluorescein, and $0.10\ F < s < 0.60\ F$ for FITC-dextran. When FITC-dextran was used as an entrapped fluorophore, f proved to be negligible. Both f and s are introduced in equations (*1*) and (*2*) as the average of duplicate measurements. The multiplication factor µ, which corrects for leakage of carboxyfluorescein from the bulk of liposomes in the incubation, is introduced in order to determine, if desired, the number of vesicles taken up by or associated with the cells. Consequently, the values obtained for Π_d and Π_i from equations (*1*) and (*2*), respectively, refer to idealized, non-leaky vesicles.

$$\mu = \frac{1}{1 - \Lambda} \qquad (3)$$

In this equation Λ stands for leakage of carobxyfluorescein from the bulk of liposomes (not associated with cells) in the incubation mixture.

$$\Lambda = \frac{1}{1 - \Lambda_o} (\Lambda_t - \Lambda_o) \quad (4)$$

Here Λ_o designates the residual fluorescence, *i.e.* the ratio of fluorescence readings before and after addition of detergent in a liposome-sample immediately after collection of the void volume of the Sephadex G-100 column. $1 - \Lambda_o$ represents the degree of self-quenching of carboxyfluorescein inside the vesicles. Usually, $\Lambda_o \approx 0.035$ (3.5%) and thus $1 - \Lambda_o \approx 0.965$ (96.5%). The residual fluorescence is most likely the result of an incomplete self-quenching of the fraction of dye molecules facing the inner monolayer of the vesicle membrane. If Λ_o is measured as a function of the carboxyfluorescein concentration inside the vesicles ($[CF]_i$), the relation $\Lambda_o = k/[CF]_i$ (5) for 10 mM < $[CF]_i$ < 100 mM is obtained. Here $k \approx 5$ mM. Λ_t stands for the leakage of carboxyfluorescein from the bulk liposomes at time t, and equals the ratio of fluorescence readings before and after addition of detergent in a sample taken from the incubation mixture. It should be noted that equation (4) gives an approximation of the leakage of carboxyfluorescein from the bulk of liposomes at a given time t, since considerable leakage of dye from the vesicles causes a decrease in the self-quenching of carboxyfluorescein inside vesicles, thus increasing the value of Λ_o. As long as $\Lambda_t < 0.2$, however, which is the case in all the experiments described, this approximation is legitimate, as can be derived from equation (5). Differences in pH between medium and intracellular compartments to which carboxyfluorescein has access, require multiplication by α. This is the factor by which, upon addition of detergent, fluorescence quantum yield increases in going from a certain intracellular pH to pH 7.4. When Zajdela cells are incubated with empty liposomes plus free carboxyfluorescein the dye is taken up by the cells, most probably mainly by diffusion across the cell membrane. After approximately 10 min of incubation the uptake of dye levels off, indicating that a steay state is established: influx = outflux. If cells are then washed several times in NaCl/Hepes buffer, and both $\bar{F}_d - \bar{s}_d$ and $\bar{F}_i - \bar{s}_i$ are determined, it is found that

$$1.7 < \frac{\bar{F}_i - \bar{s}_i}{\bar{F}_d - \bar{s}_d} < 2.0,$$

for incubation times > 10 min. Thus $1.7 < \alpha < 2.0$.

Comparison of the value of α with the increase in fluorescence quantum yield

of a carboxyfluorescein solution, which is brought from a pH = x to pH = 7.4 results in an x of 6.3 - 6.5. Thus, the average intracellular pH, at least for the compartment(s) to which the dye has access, is in the range of 6.3 - 6.5. FITC-dextran quantum yield depends in a similar manner on the environmental pH, but because the dye is not completely self-quenched inside vesicles, α cannot be applied with precision (Figs. 3 and 4).

k, finally, is a composite factor, which includes the ratios of sample- and standard fluorescence readings, sample volume corrections, etc. When carboxyfluorescein is used as an entrapped marker, the amount of dye present in vesicles adsorbed to the cell surface and/or present in endocytotic vacuoles, can be calculated: $\Pi_a = \Pi_i - \Pi_d$ (6).

Since Π_i and Π_d are calculated for idealized, non-leaky vesicles, Π_a also can be converted easily into the number of vesicles "adsorbed".

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CHAPTER III

FURTHER KINETIC CHARACTERISTICS OF *IN VITRO* INTERACTION BETWEEN LIPID VESICLES AND ZAJDELA CELLS

SUMMARY

Zajdela ascites hepatoma cells were incubated *in vitro* with small unilamellar vesicles, consisting of phosphatidylcholine, cholesterol, and phosphatidylserine in a molar ratio of 5 : 4 : 1. Phosphatidylcholine, radiolabeled in the choline moiety was used as a vesicle membrane marker. It is shown that this marker does not exchange significantly between vesicles and cells, and that - at least at short incubation times - its uptake by cells presumably represents uptake of whole vesicles. Preincubation of cells with vesicles had only a minor effect on subsequent uptake of newly added vesicles. The duration of the preincubation was found not to be important, whereas uptake of radiolabeled, carboxyfluorescein-containing vesicles was progressively, but only to a low extent, inhibited by increasing concentrations of empty, unlabeled vesicles in the preincubation. Transfer of dye from vesicles to cells was unaffected under such circumstances. Empty and carboxyfluorescein-containing vesicles were found to compete with each other for the cellular binding sites with nearly equal affinity, when present simultaneously. It is tentatively concluded that binding of vesicles to the dye-transfer sites is transient; presumably the sites are laterally cleared from vesicles. The phenomenon of cell-induced leakage interferes with the determination of the number of vesicles passively bound to adsorption sites; conclusions regarding the kinetic fate of vesicles at these sites can therefore not be drawn.

MATERIALS

RPMI 1640 tissue culture medium was purchased from Flow Labs. Inc. Egg-yolk phosphatidylcholine, cholesterol (CH-S-grade), and HEPES were from Sigma. Phosphatidylserine was isolated from bovine brain extract (Sigma) by preparative thin layer chromatography. Each lipid used gave one spot on silica gel thin layer chromatograms developed in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, by volume). [*Me*-^{14}C]- and [*Me*-^{3}H]phosphatidylcholine were prepared by remethylation

of partially demethylated egg yolk lecithin, with [^{14}C]- or [^{3}H]methyliodide (The Radiochemical Centre, Amersham, UK), essentially according to Stoffel *et al.* (1). The [^{14}C]- and [^{3}H]-labeled lecithin preparations had specific activities of 2.0 and 49.9 Ci/mol, respectively. Cells, incubated (for different lengths of time) with different concentrations of a vesicle preparation consisting of [*Me*-^{14}C]PC/[*Me*-^{3}H]PC/cholesterol/PS in a molar ratio of 2.5 : 2.5 : 4 : 1, took up both labels in equal amounts. Carboxyfluorescein (Fastman Kodak, Co., Rochester, N.Y.) was purified according to Blumenthal *et al.* (2). 100 mM stock solutions of the dye in H_2O, adjusted to pH 7.4 with 1 M NaOH, were kept refrigerated (4 °C) in the dark until use.

METHODS

Cells

Zajdela ascites hepatoma cells were isolated from adult female Wistar rats, on the fifth day after implantation, as described before (3). Cell viability was regularly checked, either with the Trypan Blue exclusion method or by measurement of LDH-leakage, according to Bergmeyer *et al.* (4), and was found to exceed 95% throughout the experiments.

Liposomes

Small unilamellar vesicles, consisting of PC/cholesterol/PS (molar ratio 5 : 4 : 1), either containing 0.135 M NaCl/0.010 M HEPES buffer (pH 7.4)* or 100 mM carboxyfluorescein, were prepared as described earlier (3).

Incubations

All incubations of cells with vesicles were carried out at 37 °C (thermostatted shaking water bath), at a cell-density of 10^7 cells/ml. The incubation medium contained no serum, and consisted of a 1 : 5 (v/v) mixture of NaCl/Hepes buffer (with or without vesicles) and RPMI 1640 medium. The latter was supplemented with 25 mM HEPES and 10 mM $NaHCO_3$, brought to pH 7.4 with 1 M NaOH. For further details on incubations, see legends to Figures. In order to assess cell-associated fluorescence or -radioactivity, the cells in the incubation mixture or in samples thereof were subjected to a standard washing procedure to remove non cell-associated vesicles: transfer to polystyrene tubes, two cycles of centrifugations (600 x g, 30s) and washes with NaCl/Hepes buffer at room temperature, transfer to fresh polystyrene tubes, one cycle

* In the following to be called: "NaCl/Hepes buffer".

of centrifugation and washing, and final uptake in 3 ml of NaCl/Hepes buffer. Radioactivity determinations were carried out with a Nuclear Chicago MkII liquid scintillation counter, equipped with a $^{3}H/^{14}C$ double-label counting facility (interchannel spillover < 5%). The scintillation mixture consisted of: up to 3.33 ml aqueous sample, 2 ml ethanol and 10 ml Hydroluma^{R} (J.T. Baker Chemicals B.V., Deventer, The Netherlands). Fluorescence measurements with a Perkin Elmer MPF-43 fluorimeter were carried out and processed as described in detail earlier (3). In experiments with carboxyfluorescein-containing vesicles, control-incubations of cells without vesicles (to assess cell-autofluorescence plus-scatter) and of cells with 50 μM free carboxyfluorescein were run. Leakage of the dye from vesicles during incubations was routinely checked and corrected for (see also Ref. 3; Appendix). For the meaning of the symbols Π_i, Π_d and Π_a, see Ref. 3; Appendix.

INTRODUCTION

In the previous chapter (3) we showed that small unilamellar vesicles, consisting of PC, cholesterol, and PS (molar ratio 5 : 4 : 1) are capable of transferring carboxyfluorescein as well as FITC-dextran into the cytoplasm of Zajdela cells, most likely via a vesicle-cell fusion-like process. Our data also suggested that during an *in vitro* incubation with cells, vesicles can become adsorbed to the cell surface, while not participating in the transfer process. These "adsorbed" vesicles seem to be highly leaky, as compared to non cell-associated ("bulk") vesicles. A number of phenomenological details concerning this "cell-induced" leakage will be dealt with in Chapter IV. This chapter gives the results of some experiments which are considered an extension of those described in Chapter II. They were designed to give an answer to an important question that arose during the experiments shown in Chapter II: is binding of vesicles to different sites at the cell surface reversible, and if so, does this reversibility result in a return of vesicles to the medium or in a migration to other regions of the cell? Although this seems to be a straightforward question we will show that efforts to find an answer to it are likely to be met by the complexity of vesicle-cell interaction.

The transfer (Π_d; see Chapter II) of carboxyfluorescein from vesicles to cells was found to level off after approximately 30 min of incubation time. (Chapter II; Fig. 1), indicating that a steady-state has been established:

dye-influx, mainly via vesicles, balances -efflux from the vesicle-cell complex* into the medium. This steady-state was observed for at least 90 min of incubation time, and was also seen by others (5,6). The establishment of a steady-state implies that either the number of transfer sites** per cell must increase with incubation time or that, in case of a fixed number of transfer sites per cell, (part of) the sites are cleared (of vesicles) at a certain rate, and subsequently become available for "new" vesicles. The steady-state is readily cancelled if its input is cut off by removal of dye-containing vesicles (Chapter II, Fig. 5): The amount of dye associated with the vesicle-cell complex rapidly declines, with a half life of about 15 min. In order to check the validity of this argumentation we studied the effects of empty*** vesicles on subsequent or simultaneous uptake of carboxyfluorescein-containing vesicles.

RESULTS

We first looked at the possibility of an exchange of radiolabeled lecithin, used as a vesicle membrane marker, between a vesicle-cell complex and non cell-associated vesicles. Cells were incubated with [^{14}C]PC labeled,PC/cholesterol/PS (molar ratio 5 : 4 : 1) vesicles. After 5 min non cell-associated vesicles were removed by subjecting the cells to the standard washing procedure (see Methods). Subsequently the cells were reincubated, either in vesicle-free medium or in medium containing unlabeled PC/cholesterol/PS (5 : 4 : 1) vesicles. At suitable times aliquots of the cell suspensions were washed (standard procedure) and assayed for cell-associated radioactivity. The result is shown in Fig. 1. Apparently, most of the [^{14}C]label, once taken up by the cells, remains cell-associated, irrespective as to whether unlabeled vesicles are present or absent. If the preincubation is extended to up to 60 min (instead of 5 min in the experiments of Fig. 1) the results are similar: once taken up by the cells, most of the label remains cell-associated. If cells are incubated for 60 min with labeled vesicles, washed (standard procedure and subsequently subjected to lipid-extraction according

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* the term "vesicle-cell complex" is to be read as short for: "cells plus cell-associated vesicles".

** the term "transfer site" is to be read as: "region of the cell surface where dye is transferred from vesicles to cells".

*** "empty" means: "non dye-containing".

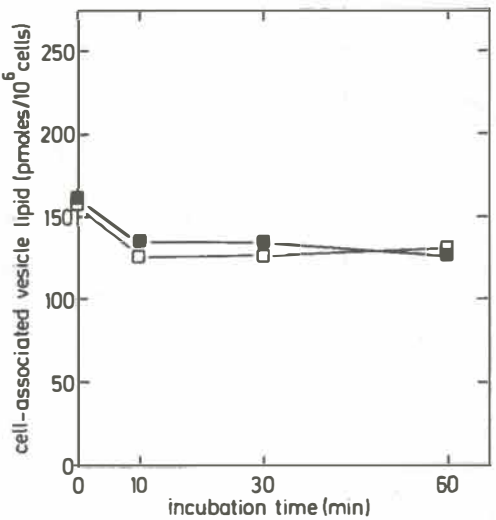

Fig. 1. *Time-dependent release of cell-associated radioactive vesicle lipid label in absence and presence of unlabeled vesicles.* 2 x 10^8 Zajdela cells were incubated (37 °C, shaking water bath) in a final volume of 20 ml in an open Erlenmeyer flask, with 1 mM (final concentration) of PC/cholesterol/PS (5 : 4 : 1) vesicles carrying 1 µCi of [*Me*-^{14}C]PC per µmole of total vesicle lipid. After 5 min of incubation the incubation mixture was divided into two equal portions, which were washed according to the standard washing procedure (see Methods). The washed packed cells were taken up in 10 ml of either vesicle-free medium, or medium containing 1 mM unlabeled PC/cholesterol/PS (5 : 4 : 1) vesicles, and incubation was continued for another 60 min. At suitable times (abscissa) duplicate 1 ml samples were withdrawn from both incubation mixtures and processed as described in the Methods section. A duplicate 50 µl sample was taken from each incubation mixture in order to assess total radioactivity. ■——■: cell-associated vesicle lipid, based on [^{14}C]PC uptake, with unlabeled vesicles present in the incubation; □——□: ditto, but in absence of unlabeled vesicles. Each point represents the average of duplicate measurements that agree within 5%.

to Bligh and Dyer (7), more than 90% of the cell-associated label is recovered as intact lecithin, the remainder mainly as lysolecithin. Hence, metabolization of the vesicle-membrane marker seems to be confined to possible de- and reacylation reactions (*cf.* 8). The result of the experiment depicted in Fig. 1 confirms some of our earlier observations (Chapter II; Fig. 5): no significant amounts of the cell-associated vesicle lipid marker or metabolites thereof are lost into the medium. It also virtually rules out the possibility of

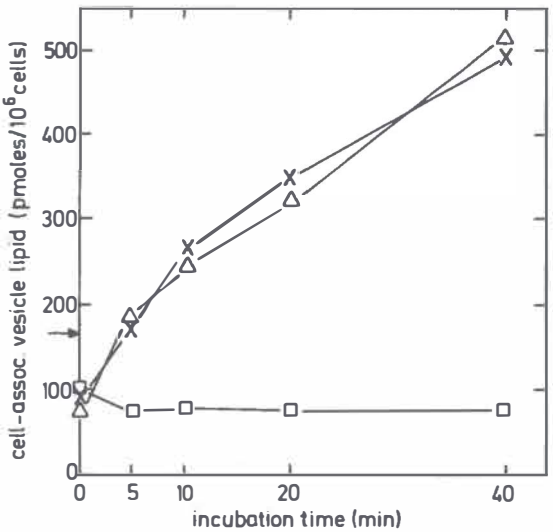

Fig. 2. *Time-dependent uptake and -exchangeability of two different vesicle lipid markers.* 1.6 x 10^8 Zajdela cells were preincubated (37 °C water bath) in a final volume of 16 ml in open Erlenmeyer flasks either with vesicle-free medium or with 0.5 mM (final concentration) of PC/cholesterol/PS (5 : 4 : 1) vesicles, carrying 10 µCi of [*Me*-^{3}H]PC per µmole of total vesicle lipid. After 5 min of preincubation a duplicate 1 ml sample was withdrawn from each mixture, processed according to the standard washing procedure (see Methods), and used for the determination of cell-associated [^{3}H]-vesicle derived lipid (arrow). The remainder of each mixture was also washed (standard washing procedure) and the packed cells of each mixture were finally resuspended in 14 ml of medium, containing 0.5 mM PC/cholesterol/PS (5 : 4 : 1) vesicles, labeled with 1 µCi of [*Me*-^{14}C]PC per µmole of total vesicle lipid. Both mixtures were then incubated for another 60 min. At suitable times (abscissa) duplicate 1-ml samples were withdrawn from each incubation mixture, processed according to the standard washing procedure, and assayed for cell-associated [^{3}H]PC- and [^{14}C]PC-label (double label counting). Duplicate 50 µl samples were taken from preincubation- and incubation mixtures in order to assess total radioactivity. □——□: cell-associated vesicle lipid, based on [^{3}H]PC uptake; X——X: cell-associated vesicle lipid, based on [^{14}C]PC uptake; Δ——Δ: cell-associated vesicle lipid, based on [^{14}C]PC uptake, after preincubation with vesicle-free medium.

a significant exchange of either labeled whole vesicles or labeled lecithin (plus metabolites thereof) between vesicle-binding sites at the cell-surface and non cell-associated vesicles in the medium. Hence, use of radiolabeled PC as a vesicle-mebrane marker apparently does not lead to an underestimation of the amount of cell-associated vesicle lipid. The results strongly suggest a

one-way traffic for PC/cholesterol/PS (5 : 4 : 1) small unilamellar vesicles in an incubation with cells: towards the cells. Fig. 2 shows that time-dependent uptake of [^{14}C]PC-labeled vesicles apparently is not significantly affected by a preincubation with [^{3}H]PC-labeled vesicles, and that, after removal of bulk [^{3}H]-vesicles, most of the [^{3}H]PC-label remains cell-associated, much the same as in Fig. 1. In Fig. 2 the arrow (left ordinate) indicates the amount of [^{3}H]-vesicle lipid found cell-associated after the cells had been subjected to the standard washing procedure in order to remove bulk [^{3}H]-vesicles and to replace them by [^{14}C]-vesicles. For the determination of the amount of cell-associated [^{14}C]-vesicle lipid aliquots of the incubation mixture had to be washed (standard procedure) again. Such excessive washing most likely removes some loosely cell-associated [^{3}H]-vesicles from the cell-surface (see also Chapter IV). The standard washing procedure is considered effective (and necessary) for removal of virtually all non cell-associated vesicles: it results in a more than 10^{5}-fold dilution of the bulk vesicle concentration, while leaving at the same time 0.2 - 2.0% (depending on vesicle lipid concentration and incubation time; see also Chapter II) of bulk vesicles to be found cell-associated. The washing procedure may, however, lead to an underestimation of the amount of vesicle lipid that is actually (albeit loosely) cell-associated. In preliminary experiments Scatchard plots of concentration-dependent uptake of [^{3}H]PC-labeled, PC/cholesterol/PS (5 : 4 : 1) vesicles show that especially at higher (> 0.1 mM lipid) vesicle concentrations substantial amounts of vesicle lipid become only loosely cell-associated. Nevertheless we observed that when at the end of a preincubation bulk vesicles were removed less effectively, the kinetic fate of the lipid label was essentially similar; the only difference is that the amounts of [^{14}C]-vesicle lipid in Fig. 1 and [^{3}H]-vesicle lipid in Fig. 2, which remain cell-associated, are 30-40% higher in the entire incubation time-range studied.

In order to assess how and to what extent uptake of vesicles might depend on the concentration of vesicles in a preincubation, we preincubated cells with different concentrations of [^{3}H]PC-labeled vesicles for 5 min, removed the medium and replaced* it by medium containing a fixed concentration of

* Since, in this experiment, we did not have to assess the amount of vesicle lipid which was cell-associated at the end of the preincubation, the medium could simply be replaced. The minimal mol ratio of [^{14}C]- over [^{3}H]-vesicles left behind in the incubation was estimated to be 13.

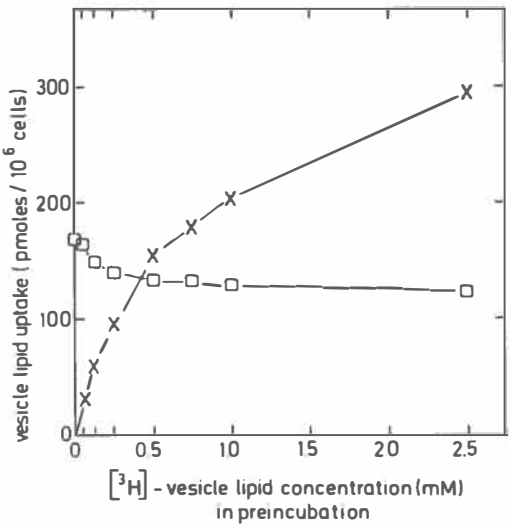

Fig. 3. *Inhibition of uptake of* [^{14}C]*PC-labeled vesicles as a result of a preceding incubation with different concentrations of* [^{3}H]*PC-labeled vesicles.* A series of duplicate preincubations (37 °C, shaking water bath) was set up Each (open) polystyrene tube contained 1.1 x 10^7 Zajdela cells together with a suitable dilution (for concentrations see abscissa) of one vesicle preparation, consisting of PC/cholesterol/PS (5 : 4 : 1) carrying 25 µCi of [*Me*-^{3}H]PC per µmole of total vesicle lipid, in a final volume of 1.1 ml. After 5 min, 6 ml of NaCl/Hepes buffer was added to each tube and the cells were quickly spun down (600 x g, 30s). The supernatants were removed, leaving approximately 100 µl of packed cells in each tube. Subsequently, to each tube 1 ml of medium containing 0.55 mM of PC/cholesterol/PS (5 : 4 : 1) vesicles carrying 1 µCi of [*Me*-^{14}C]PC per µmole of total vesicle lipid was added, and the cells were resuspended by gentle vortexing. Incubation was continued for another 5 min. The contents of each tube were then washed according to the standard washing procedure (see Methods); the washed cells were assayed for cell-associated [^{3}H]PC- and [^{14}C]PC-radioactivity (double label counting). Duplicate 50 µl samples were taken from preincubation- and incubation mixture in order to assess total radioactivity. X——X: cell-associated vesicle lipid, based on [^{3}H]PC uptake; □ ——□: cell-associated vesicle lipid, based on [^{14}C]PC uptake. Each point represents the average of measurements (agreeing within 10%) from duplicate incubations.

[^{14}C]PC-labeled vesicles. Subsequently, incubation was continued for another 5 min and cell-associated [^{3}H]- and [^{14}C]-radioactivities were determined. The results are shown in Fig. 3. With 2.5 mM [^{3}H]-vesicles in the preincubation, 28% inhibition of the uptake of 0.5 mM [^{14}C]-vesicles is found, suggesting that, in case of a fixed total number of sites per cell, most of these sites are cleared rapidly and become available to bind "new" vesicles. If in similar experiment the preincubation-medium is not simply replaced by medium containing [^{14}C]-vesicles, but, instead, cells are given the standard washing procedure, the values of [^{3}H]-vesicle uptake are on the average 30% lower when compared to the values of Fig. 3. The corresponding [^{14}C]-vesicle uptake curve remains unchanged. The latter finding suggests that those [^{3}H]-vesicles which are removed from their cellular binding sites do not take part significantly in the inhibition of subsequent [^{14}C]-vesicle uptake. The experiment of Fig. 3 was also done with a trace of cholesteryl [1-^{14}C]oleate (instead of [^{14}C]PC) as a vesicle marker: a similar [^{14}C]-label uptake curve was obtained, indicating the absence of PC-exchange under these conditions. Cholesteryl oleate is considered non-exchangeable between membranes (9).

Next, we studied the effects of a preincubation with empty vesicles on uptake of carboxyfluorescein-containing vesicles, using essentially the same experimental set-up as was chosen for the experiment of Fig. 3. Preincubation was done with empty, unlabeled vesicles, and the incubation with [^{14}C]PC-labeled vesicles containing 100 mM carboxyfluorescein. The results are shown in Fig. 4. Vesicle-uptake, based upon [^{14}C]PC uptake* appears to be inhibited in much the same way as in the experiment of Fig. 3. Transfer of dye from vesicles to cells (Π_d; see Methods) is not significantly affected. The amount of total cell-associated dye (Π_i) and "adsorbed"** carboxyfluorescein (Π_a) first increases with increasing empty vesicle concentration in the preincubation, to a maximum at about 0.10 mM empty vesicles, and then gradually decreases in a way similar to the amount of cell-associated vesicle lipid. This course of dye-"adsorption" is difficult to interpret. The experiments described in Chapter II led us to conclude that, most likely, vesicles which are

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* As in the experiment of Fig. 3, use of Cholesteryl [1-^{14}C]oleate resulted in a similar inhibition curve.

** To be read as short for "dye which is cell-associated inside vesicles, in a highly quenched state".

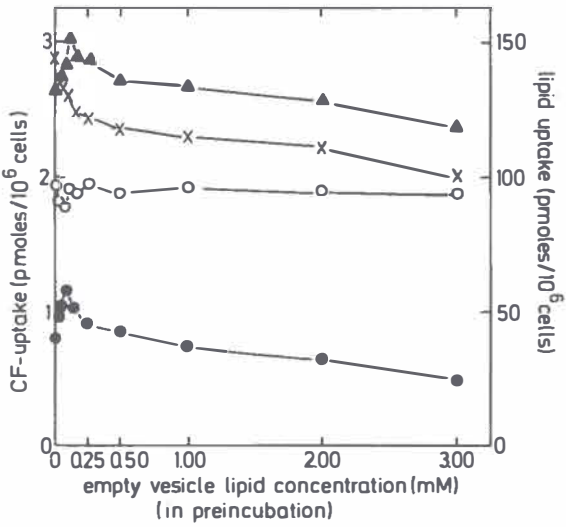

Fig. 4. *Inhibition of uptake of vesicle-entrapped carboxyfluorescein and of vesicle lipid as a result of preceding incubation with different concentrations of empty, unlabeled vesicles.* A series of duplicate preincubations (37 °C, shaking water bath) was set up. Each (open) polystyrene tube contained 1.2 x 10^7 Zajdela cells together with a suitable dilution (for concentrations see abscissa) of one empty PC/cholesterol/PS (5 : 4 : 1) vesicle preparation, in a final volume of 1.2 ml. After 5 min, 6 ml of NaCl/Hepes buffer was added to each tube and the cells were quickly spun down (600 x g, 30s). The supernatants were removed, leaving approximately 100 µl of packed cells in each tube. Subsequently, to each tube 1.1 ml of medium containing 0.55 mM of PC/cholesterol/PS (5 : 4 : 1) vesicles carrying 1 µCi of [*Me*-^{14}C] PC per µmol of total vesicle lipid was added, and the cells were resuspended by gentle vortexing. Incubation was continued for another 5 min. The contents of each tube were then washed according to the standard washing procedure (see Methods); the washed cells were finally suspended in 3 ml of NaCl/Hepes buffer. Directly- and indirectly measurable fluorescence was read, and cell-associated [^{14}C]PC-radioactivity determined. Duplicate 50 µl samples were taken from each incubation mixture to determine total radioactivity and leakage of carboxyfluorescein from bulk vesicles. X——X: cell-associated vesicle lipid, based on [^{14}C]PC uptake; ▲——▲: total cell-associated carboxyfluorescein, as measured after addition of detergent (Π_i, see Methods); O——O: cell-associated carboxyfluorescein, as measured before addition of detergent (Π_d, see Methods); ●——●: "adsorbed" carboxyfluorescein (Π_a, see Methods). Each point represents the average of measurements (agreeing within 10%) from duplicate incubations. CF: carboxyfluorescein.

simply adsorbed to the cell-surface (*i.e.* not taking part in transfer of dye), are highly leaky. Such cell-induced leakage of carboxyfluorescein from vesi-

cles obviously will lead to an underestimation of the number of "adsorbed" vesicles (expressed by Π_a). Presumably, this underestimation is (partly) undone by the presence (as a result of the preincubation) of empty vesicles at the cell-surface. Consequently, the observed degree of inhibition of Π_a (and of Π_i) would be understimated. Even without taking into account this probable underestimation, we tend to conclude that the transfer- and "adsorption"-component are kinetically distinguishable. Other experiments (not shown) indicate that extension of the duration of the preincubation does not result in different degrees of inhibition of the uptake parameters. Hence we tentatively conclude that, in case of a fixed total number of transfer sites per cell, such sites are cleared rapidly.

So far, we have looked at the effects of a *pre*incubation with vesicles. In order to investigate whether empty vesicles compete with simultaneously present carboxyfluorescein-containing vesicles for cellular binding sites, we incubated cells with mixtures of dye-containing [^{14}C]PC-labeled vesicles, at a fixed concentration, and empty unlabeled vesicles, at variable concentrations. The results are depicted in Fig. 5. [^{14}C]-vesicle lipid uptake, dye-transfer, and total cell-associated dye are found to be progressively blocked with increasing empty vesicle concentration: at an 8-fold excess of empty- over dye-containing vesicles, inhibition amounts to 69%, 60%, and 55%, respectively, for the 3 parameters, taking the corresponding values obtained in the absence of empty vesicles as 100 percent. Uptake of "adsorbed" vesicle-carboxyfluorescein (Π_a) is inhibited by maximally 43%; inhibition levels off with empty vesicle lipid-concentration exceeding 0.5 mM. Presumably the effect of the presence of empty vesicles on the value of Π_a is of similar nature as proposed in discussing the result of Fig. 4: most likely the leak-inducing adsorption sites at the cell-surface are partly occupied by empty vesicles, leading to an underestimation of the degree of competitive inhibition of Π_a by empty vesicles. The magnitude of underestimation would - obviously - be inversely correlated to the empty vesicle concentration (see also Chapter IV). The molar ratio of total cell-associated dye to total cell-associated vesicle lipid can be calculated from Fig. 5; it increases from 0.015 (no empty vesicles present) to 0.021 (at 4 mM empty vesicles), indicating progressive abolishment of the expression of cell-induced leakage of carboxyfluorescein from vesicles. If in the experiment of Fig. 5, empty plus dye-containing vesicles are replaced by dye-containing vesicles

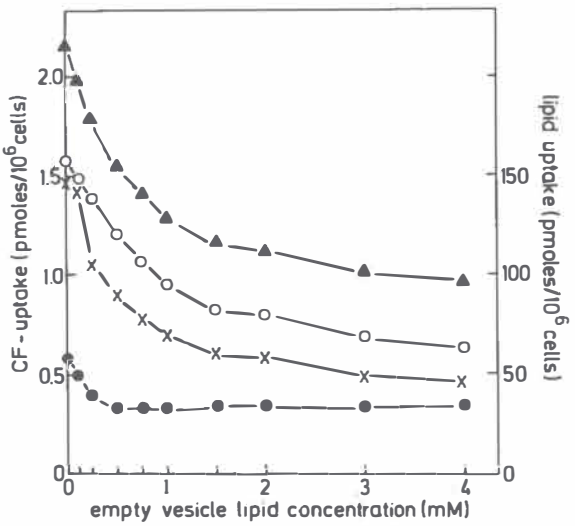

Fig. 5. *Inhibition of uptake of vesicle-entrapped carboxyfluorescein and of vesicle lipid by different concentrations of simultaneously present empty, unlabeled vesicles.* A series of duplicate incubations (37 °C, shaking water bath) was done. Each (open) polystyrene tube contained (in a final volume of 1.2 ml) 1.2 x 10^7 Zajdela cells together with a mixture of empty, unlabeled- plus 100 mM carboxyfluorescein-containing PC/cholesterol/PS (5 : 4 : 1) vesicles, carrying 1 µCi of [*Me*-^{14}C]PC per µmole of total vesicle lipid. Dye-containing, labeled vesicles were present in all tubes at a fixed final concentration of 0.5 mM; empty, unlabeled vesicles were present at variable concentrations (see abscissa). After 5 min of incubation each tube was subjected to the standard washing procedure (see Methods); the washed cells were finally taken up in 3 ml of NaCl/Hepes buffer. Directly- and indirectly measurable fluorescence was read (see Methods) and cell-associated [^{14}C]PC-radioactivity determined. Duplicate 50 µl samples were taken from each incubation mixture to determine total radioactivity and leakage of carboxyfluorescein from bulk vesicles. X——X: cell-associated vesicle lipid, based on [^{14}C]PC uptake; ▲——▲: total cell-associated carboxyfluorescein, as measured after addition of detergent (Π_i; see Methods); O——O: cell-associated carboxyfluorescein, as measured before addition of detergent (Π_d, see Methods); ●——●: "adsorbed" carboxyfluorescein (Π_a, see Methods). Each point represents the average of measurements (agreeing within 10%) from duplicate incubations. CF: carboxyfluorescein.

only, the values of the uptake parameters are nearly restored (within 10 min) to the corresponding values of Fig. 5, at zero empty vesicle concentration. This indicates that the inhibition of uptake of carboxyfluorescein-containing

vesicles by empty vesicles is highly competitive. It should be emphasized that a nearly complete competitive inhibition of transfer, as reported by Blumenthal *et al.* (2), can only be observed if one applies a very large excess (> 50 fold) of empty vesicles over dye-containing vesicles. In our experimental setting the upper limit of the ratio empty/full was dictated by two restraints: firstly, at a total vesicle lipid concentration exceeding 5 mM, cell viability (see Methods) starts to decline quickly. Secondly, less then 0.1 pmole of carboxyfluorescein per 10^6 cells cannot be measured with sufficient accuracy. Laser flow microfluorimetric systems as employed by Blumenthal *et al.* (2) for the measurement of carboxyfluorescein-transfer, are endowed with much greater sensitivity: a few thousand dye-molecules per cell can easily be detected. These systems offer a number of additional advantages over conventional fluorimetry, such as: simultaneous assessment of cell viability and on-line cell-population statistical analysis.

For a correct interpretation of the data of Fig. 5 it is necessary to assess whether empty and dye-containing vesicles have equal or different affinity for the cellular binding sites. In order to investigate this, we incubated cells with a fixed total number of vesicles, consisting of empty- and full vesicles at a variable ratio: the total number of vesicles to be found cell-associated is then expected to be constant. The result of the experiment is given in Fig. 6. The curves A_1 and A_2 represent vesicle-concentration dependent (no empty vesicles present) dye-transfer (Π_d) and "adsorbed" dye (Π_a), respectively, and serve as internal controls. Both transfer and "adsorption" are found to be about linearly increasing (curve B_1 and B_2, respectively) with decreasing empty/full vesicle lipid ratio. The dashed lines indicate the theoretical relation between dye-uptake and empty/full vesicle ratio, in case empty and dye-containing vesicles are to display equal affinity for the cellular binding sites. Curve B_1 nearly coincides with its "iso-affinity" line. For curve B_2 there seems to be a deviance. We tend to interpret this as to result from the interference of cell-induced leakage of vesicle-contents with the determination of the amount of "adsorbed" dye. This problem has been discussed above and in Chapter II. With decreasing empty/full vesicle ratio such induced leakage is likely to be progressively unmasked. Consequently, empty and dye-containing vesicles can be regarded to possess equal affinity for the cellular binding sites.

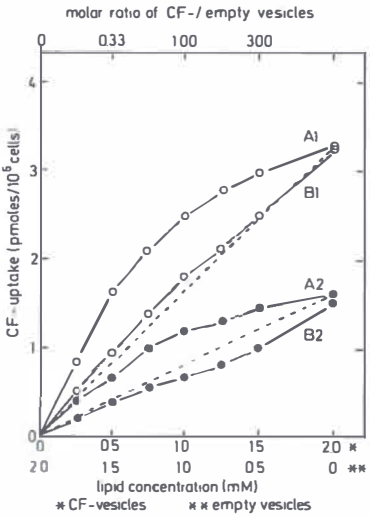

Fig. 6. *Transfer and "adsorption" of carboxyfluorescein with mixtures of full and empty vesicles at a fixed total vesicle-concentration.* Two sets of duplicate incubations (37 °C, shaking water bath) were performed. Each (open) polystyrene tube contained 1.1 x 10^7 Zajdela cells in a final volume of 1.1 ml. The tubes of set A (going with curves A) contained different concentrations (see abscissa, *) of PC/cholesterol/PS (5 : 4 : 1) vesicles with 100 mM carboxyfluorescein entrapped. The tubes of set B (going with curves B) contained a fixed amount (final total concentration 2 mM) of empty- (see abscissa, **) plus carboxyfluorescein-vesicles, at different ratios (indicated on the abscissa at the top of the graph). After 5 min of incubation the mixtures were processed according to the standard washing procedure (see Methods); the washed cells were finally suspended in 3 ml of NaCl/Hepes buffer. Directly- and indirectly measurable fluorescence was read (see Methods). Duplicate 50 µl samples were taken from each incubation mixture to determine leakage of carboxyfluorescein from bulk vesicles. O——O: cell-associated carboxyfluorescein, as measured before addition of detergent (Π_d, see Methods); ●——●: "adsorbed" carboxyfluorescein (Π_a, see Methods). -----: see text. Each point represents the average of measurements (agreeing within 10%) from duplicate incubations. CF: carboxyfluorescein.

DISCUSSION

Data presented in this report indicate that for Zajdela cells radiolabeled lecithin, used as a marker of small unilamellar PC/cholesterol/PS (5 : 4 : 1) vesicles is largely unexchangeable between an existing vesicle-cell complex and non cell-associated vesicles. This conclusion is mainly based on the experiments depicted in Figs. 1-3: once taken up by the Zajdela cells, the

radiolabeled lecithin for the greater part remains associated with the cells. The possibility of transfer or exchange of lecithin between vesicles and cells has been considered in a number of studies (6,10-14). The work of Pagano and Huang (13) on the *in vitro* interaction of dioleoyllecithin vesicles with Chinese hamster V79 cells, and of Huang *et al.* (12) on uptake of [^{14}C]-labeled egg-PC by mouse thymocytes indicated that at 2 ^{o}C (in contrast to 37 ^{o}C) a substantial part of lecithin uptake was found to be accounted for by exchange with cellular PC. In an elegant study, Sandra and Pagano (15) demonstrated that this exchange at low temperature consists of a one-for-one exchange between the outer leaflets of vesicle- and plasma membrane. In a recent study on the interaction of isolated hepatocytes with PC/cholesterol/DCP (2 : 1.5 : 0.22) small unilamellar vesicles, Hoekstra *et al.* (6) were able to demonstrate that at 37 ^{o}C radiolabeled PC is transferred/exchanged to a considerable extent between vesicles and cells, in contrast to cholesteryl [1-^{14}C]oleate, which was also used as a vesicle membrane marker. For Zajdela cells we showed (Chapter II, Fig. 6) that at short incubation times vesicle lipid uptake values based upon uptake of cholesteryl ester roughly match those based upon PC, indicating that at short incubation times transfer/exchange of PC between vesicles and Zajdela cells cannot account for a significant part of measured [^{14}C]PC uptake.

With regard to the result of the experiment shown through Fig. 1, we have to ask whether the radiolabeled lecithin that remains cell-associated represents vesicles sticking to the cells or molecularly transferred lipid. There is, at least in theory, the possibility that vesicles make a transient contact with the cell-surface, donate (radioactive) PC to it, and return to the medium. It can furthermore not be excluded that transfer of vesicle-contents into the cells could be (completely) accomplished within such a short, momentarily contact. In case such a "hit and run" mechanism would be operative, a measured time- or vesicle-concentration-dependent increase of cell-associated radioactive lecithin would not represent an increase of the number of vesicles actually cell-associated at the time of measurement. There are some arguments against an exclusive involvement of such a "hit and run" mechanism. First, use of carboxyfluorescein as a vesicle-entrapped marker allows to assess the presence (especially after short incubation times) of a substantial number of vesicles in persistent association with the cells (this report, Figs. 4-6; Chapter II; Refs. 2 and 6). Most likely, the occurrence of vesicle-

leakage induction by the cells (see Chapters II and IV) leads to a fair underestimation of this number of cell-associated vesicles. Hence, a substantial part of the radiolabled PC which is found cell-associated will be accounted for by "adsorbed" vesicles. Second, we consider it highly unlikely that, in case of such a hypothetical, transient vesicle-cell contact, transfer of cholesteryl [1-^{14}C]oleate to the cells would occur at a rate similar to that of radiolabeled lecithin. In the experiments shown in Figs. 3 and 4 the measured uptake of radiolabeled lecithin was similar to that of cholesteryl [1-^{14}C]-oleate, used in trace amounts as a vesicle membrane marker. Third, if vesicles would donate a considerable amount of their lecithin to the cell surface without concomitant replacement of these molecules via exchange with lecithin from the plasma membrane, it would seem highly unlikely that the vesicular structure would be preserved, both for geometrical reasons (the vesicle diameter cannot be reduced very much because of packing constraints) as well as for reasons of lipid composition since vesicles with phospholipid : cholesterol ratios less than 1 can probably not exist (*cf.* 16). Fourth, the finding (Fig. 4) that radioactive label uptake and dye transfer are not equally inhibited by preceeding presence of empty vesicles argues against a complete parallelism of dye-transfer and vesicle lipid uptake. We therefore tend to conclude that - for short incubation times - the major part of cell-associated radiolabeled PC represents actually cell-associated vesicles, either transferring their contents into the cell or being adsorbed without taking part in transfer. The most straightforward interpretation of the data presented in Fig. 4 would therefore be, that - following a preincubation with various amounts of empty vesicles - the transfer sites either have been cleared laterally from empty vesicles and/or that the total number of transfer sites per cell has increased during the preincubation with empty vesicles. At present we cannot unambiguously indicate which of the latter two possibilities comes closest to reality. In a formal sense, *i.e.* for calculations on vesicle-cell interaction kinetics, it should not make a difference whether the first or the second possibility prevails: in either case the dye-transfer process could be characterized by a certain site-clearance rate constant (*cf.* Ref. 2). However, if at a fixed incubation time the total number of transfer sites per cell would increase as a function of the total vesicle lipid concentration, we would not expect to observe a competitive inhibition of dye-transfer by increasing amounts of simultaneously present empty vesicles as is found in the experi-

ments shown in Figs. 5 and 6 (*cf.* also Ref. 2). The finding that the duration of a preincubation with vesicles does not affect the degree of inhibition of the uptake parameters (including dye-transfer) argues against an incubation-time dependent increase of the number of transfer sites per cell. We therefore assume that, most likely, the number of transfer sites per cell is constant within the ranges of incubation time and vesicle lipid concentration explored. A great deal of additional careful experimentation including a study on the incubation-time dependency of the competition between empty- and dye-containing vesicles for the different sites at the cell surface, will be necessary to give a decisive solution to the problem stated above. Likewise, the question raised in the Introduction cannot be fully answered: assuming that a fixed number of transfer sites is cleared from vesicles at a certain rate, we do not know with certainty the destination of the "consumed" vesicles; back into the medium (less likely, Fig. 1) or to other regions of the cell(-surface). Alternatively, one could imagine the physical reversal of a transfer site clearance to happen: cell-associated vesicles could be cleared from transfer sites, *i.e.* a cell-surface region serving as a transfer site could migrate by lateral flow of membrane-components to another part of the cell-surface, constituting a new transfer site. From a related point of view, it would be of interest to know how vesicle-dye transfer responds to manipulation of the cytoskeletal system, as the latter is known to play an important role in the organization of cell-surface components (17).

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C H A P T E R IV

CELL-INDUCED LEAKAGE OF LIPOSOME CONTENTS

SUMMARY

Using the principle of relief of self-quenching of carboxyfluorescein [Weinstein, J.N. *et al.* (1977) Science 195, 489-492] upon leakage of the dye from the interior of lipid vesicles, we investigated the integrity of sonicated small unilamellar vesicles in the presence of isolated hepatocytes, Zajdela ascites hepatoma cells, and plasma membranes of either cell type. We observed that cells as well as plasma membranes induce leakage of carboxyfluorescein from vesicles. Two parameters (initial rate - and maximal level of induced leakage) were determined to quantitate the leakage events, and were found to depend on cell density, vesicle concentration and vesicle lipid composition. The magnitude of both parameters is shown to increase with cell density, and to decrease with increasing vesicle lipid concentration, and seems to be correlated to the number of vesicles found in close contact with the cell. For vesicles made of phosphatidylcholine and cholesterol, the degree of induced leakage increases steeply with cholesterol-contents increasing from 30 to 40 mole %. In case of simultaneous presence of 10 mole % phosphatidylserine, induced leakage is observed at cholesterol-contents exceeding 20 mole %. We show that leak-inducing activity resides in the plasma membrane, and that it can be considerably reduced by treatment of the plasma membranes with neuraminidase or trypsin, suggesting the involvement of cell-surface glycoprotein(s). Release of activity from intact cells and isolated plasma membranes into the medium occurs spontaneously (at a slow rate) but can be facilitated by freezing and thawing; the activity can subsequently be recovered in a soluble form from the medium. The different degrees of leak-induction observed on vesicles of different lipid composition are discussed in terms of accessibility of the vesicle membrane to cell-surface proteins, inhomogeneity of vesicle populations, and possible alterations of the physical properties of vesicles approaching the cell surface.

MATERIALS AND METHODS

Materials

Egg yolk lecithin, cholesterol (CH-S grade) and dicetylphosphate were from Sigma. Phosphatidylserine was purified from bovine brain extract (Sigma) by preparative thin layer chromatography. [N-methyl-^{3}H] egg yolk lecithin (49 μCi/μmol) was prepared essentially as described by Stoffel (1975). All lipids used exhibited one spot on TLC in $CHCl_3/CH_3OH/H_2O$ (65:25:4).

Horse radish peroxidase (HRPO), trypsin (from bovine pancreas) and the diammonium salt of 2,2'-azino di (3-ethylbenzthiazolin) sulphonic acid (ABTS) were bought from Boehringer, Mannheim, G.F.R. Neuraminidase (Clostridium perfringens), specific activity 5.4 Units/mg protein using NAN-lactose, was obtained from Sigma. Trypsin inhibitor (from Soybean) was purchased from Merck. Carboxyfluorescein (CF) was from Eastman Kodak and purified as described by Blumenthal *et al.* (1977). FITC-dextran (average M.W. 3000) was purchased from Pharmacia, and HEPES from Sigma.

Preparation of phospholipid vesicles

Small unilamellar vesicles of various lipid compositions and molar ratios (given in the appropriate figure), and containing either 100 mM carboxyfluorescein (in double distilled water, adjusted to pH 7.4 with NaOH) or horse radish peroxidase (50 mg/ml) in 0.135 M NaCl/0.01 M HEPES, pH 7.4, were generated as described elsewhere (Van Renswoude *et al.*, 1979; Hoekstra *et al.*, 1978). Non-entrapped substance was removed by column gel chromatography (Sephadex G-100 for CF-containing vesicles, and Sepharose CL-6B for those containing HRPO). Vesicle lipid concentrations were assessed by lipid phosphorus determination (Chen *et al.*, 1956).

Cells

Hepatocytes and Zajdela cells were isolated as described previously (Hoekstra *et al.*, 1978; Van Renswoude *et al.*, 1979) and kept in calcium free Hanks' Balanced Salt Solution before use in experiments.

Isolation of plasma membranes

The isolation of plasma membranes, derived from isolated hepatocytes and Zajdela cells, was carried out according to the method of Ray (1970). Purity of plasma membrane fractions (d = 1.16 - 1.18 g/cm^3) was assessed by enzyme determination of 5'-nucleotidase (Aronson *et al.*, 1974), glucose-6-phosphatase

(Swanson, 1955) and acid phosphatase (Gianetto and De Duve, 1955). Specific activities of 5'-nucleotidase in hepatocyte- and Zajdela plasma membrane fractions were 10- and 15-fold enriched, respectively, when compared to cell-homogenates. In plasma membrane fractions of neither cell-type acid phosphatase activity could be detected, whereas only trace amounts of glucose-6-phosphatase were present.

Measurements of vesicle-leakage induced by cells or plasma membranes

Continuous monitoring of leakage of CF from vesicles was carried out with a flow chart recorder coupled to a Perkin Elmer MPF43 fluorescence spectrophotometer, set at excitation and emission wavelengths of 490 and 520 nm, respectively.

Incubations in which CF-leakage from vesicles was continuously monitored were carried out in 1 cm light-path quartz cuvettes fitted in a thermostatted (37 ^{o}C) sample holder inside the fluorescence spectrophotometer. Calibration of the instrument was done as described elsewhere (Van Renswoude *et al.*, 1979).

HRPO-containing vesicles were incubated in 0.135 M NaCl/0.010 M HEPES, pH 7.4, at 37 ^{o}C (thermostatted shaking water bath), in polystyrene tubes. At suitable time intervals 0.5 ml samples were withdrawn from the incubation mixtures and centrifuged during 30 sec in an Eppendorf table top centrifuge. The clear supernatants were assayed for enzyme activity in absence and presence of Triton X-100, essentially according to Steinman and Cohn (1972), with ABTS as an oxygen acceptor.

Other methods

Protein was determined by the method of Lowry *et al.* (1951). Cell viability, monitored by exclusion of Trypan Blue (0.25%), was > 95% throughout the experiments. Cell-associated radioactivity, after incubation with [N-methyl-^{3}H] egg PC containing vesicles, was determined as described before[Hoekstra *et al.*, (1978); Van Renswoude *et al.*, (1979)].

INTRODUCTION

Lipid vesicles* are currently under study as potential carriers for various water-soluble substances to be introduced into mammalian cells (for reviews see Pagano and Weinstein, 1978 and Tyrrell *et al.*, 1976). Yet, many of the

* In this report, the terms (lipid)vesicle and liposome are used as synonyms.

details of vesicle-cell interaction, particularly at the level of the cell-surface, are unknown. Recent studies from our laboratory (Van Renswoude *et al.*, 1979 and Hoekstra *et al.*, submitted) as well as from others (Szoka *et al.*, 1979) called attention to the probability of leakage of vesicle-entrapped substances as a result of contact between vesicles and cell-surfaces. Szoka *et al.* (1979) recognized, that the presence of cells enhanced leakage of the fluorophore carboxyfluorescein (CF) from vesicles. In studying the interaction of vesicles with Zajdela ascites hepatoma cells (Van Renswoude *et al.*, 1979), we obtained evidence of CF-release from vesicles in presence of cells, superimposed on bulk leakage, and we arrived at the conclusion that this superimposed CF-leakage most likely occurs from a fraction of vesicles that is in actual physical contact with the cells. A similar superimposed leakage of CF was observed by Hoekstra *et al.* (submitted) in their study of *in vitro* interaction of isolated hepatocytes and lipid vesicles. Since this leakage-phenomenon was observed with various cell-types as well as with vesicles of various lipid compostion, we considered it important to have more insight into the nature of its mechanism. We used CF as an entrapped substance mainly for three reasons: first, leakage of CF from vesicles can easily be monitored continuously; second, there is no need for separation of vesicles from released solute and third, because of the high sensitivity of CF-fluorescence measurement, the use of this dye offers the possibility to investigate the effect at relatively low vesicle concentrations.

The CF-method has been described in detail (Weinstein *et al.*, 1977 and Blumenthal *et al.*, 1977). Briefly, the dye is entrapped inside lipid vesicles in a high, self-quenched, concentration (100 mM). Escape of CF from the inner aqueous compartment of a small unilamellar vesicle results in a vast dilution of the dye into the surrounding medium, causing an instantaneous fluorescent signal (F_{direct}). The total amount of CF within the test system can be assessed by the addition of a detergent (*e.g.* Triton X-100) which causes immediate and complete disintegration of the vesicles, concomitant dilution of all CF into the total system volume, and a maximal fluorescent signal ($F_{indirect}$). Thus, leakage of CF from vesicles can be expressed in terms of F_d/F_i. Details on the quantitative treatment of CF-leakage data are given elsewhere (Van Renswoude *et al.*, 1979).

RESULTS AND DISCUSSION

Description of the test system

Fig. 1 shows the typical time course of the F_d/F_i ratio (in the following to be designated as Λ) recorded on small unilamellar vesicles, composed of PC, cholesterol, and PS (molar ratio 5 : 4 : 1), containing 100 mM carboxyfluorescein. Section A represents basal (or bulk-) leakage of carboxyfluorescein from vesicles in the absence of cells. Curve B results from the addition (arrow) of either hepatocytes or Zajdela cells. The intersection C then represents the jump in fluorescence quantum yield, $\Delta F_d/F_i = \Delta\Lambda$, due to the presence of cells during a certain time and expresses the induced leak of carboxyfluorescein from vesicles. For all vesicle lipid compositions as well as for all cell-densities and vesicle lipid concentrations employed, curve B

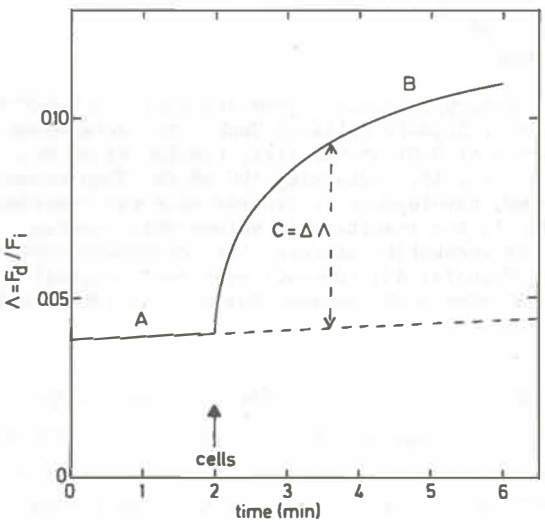

Fig. 1. *Schematic representation of a recorder fluorescence tracing of carboxyfluorescein leakage from vesicles.* Section A: basal (bulk-) leakage of dye from vesicles in the absence of cells. Curve B: development of the fluorescent signal upon addition of cells (arrow). Intersection C: increase of fluorescence quantum yield due to the presence of cells, as a function of time. The units along the Y-axis were determined after addition of Triton X-100 [final concentration 1% (v/v)]. Symbols: F_d: (direct) fluorescence (arbitrary units) in absence of detergent. F_i: (indirect) fluorescence (arbitrary units) in presence of detergent. Λ: ratio of F_d to F_i. For further details see text.

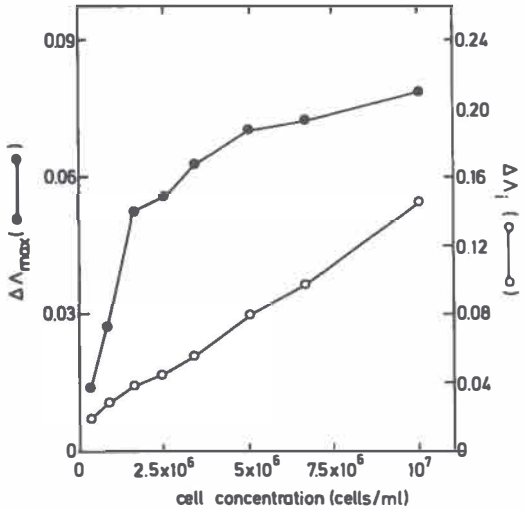

Fig. 2. *Cell-induced leakage of carboxyfluorescein from vesicles as a function of cell density.* Different numbers of Zajdela cells in Hanks' BSS were added to a fixed final lipid concentration of 0.01 mM vesicles, consisting of PC, cholesterol and PS (molar ratio 5 : 4 : 1), containing 100 mM CF. Final volume: 3 ml. For each cell density studied, development of fluorescence was recorded as described in the legend to Fig. 1; the resulting $\Delta\Lambda$ values were plotted against the density of cells in the incubation mixture. For incubation conditions see Materials and Methods. Symbols: $\Delta\Lambda_i$ (O——O) represents initial rate of dye leakage. $\Delta\Lambda_{max}$ (●——●) represents maximal value of $\Delta\Lambda$ (see legend of Fig. 1).

levels off with time and after approximately 10 min attains the same slope as line A. The intersection C then equals $\Delta\Lambda_{max}$. $\Delta\Lambda$ does not change upon removal of the cells, indicating that at any incubation time and at the vesicle concentration used, transfer of dye from vesicles to cells or from the medium (after leakage from vesicles) to cells, is negligible. For incubation times between 3 and 10 min, plots of $(\Delta\Lambda)^{-1}$ *vs.* time^{-1} yield straight lines and thus allow precise determination of $\Delta\Lambda_{max}$. The initial rate of induced leakage (*i.e.* immediately after addition of cells to the system) is obtained by construction of the tangent to curve B at t = 0 (addition of cells) and will be denoted as $\Delta\Lambda_i$ (fractional leak per min).

Influence of cell type and cell-density

Hepatocytes and Zajdela cells give similar patterns of induced leakage,

although it requires 6 (3) times as many Zajdela cells as hepatocytes to produce a certain $\Delta\Lambda_{max}$ ($\Delta\Lambda_i$).

For a fixed concentration of vesicles, the values of both $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ depend on the density of cells in the incubation mixture, as is shown in Fig. 2. $\Delta\Lambda_{max}$ tends to level off with increasing cell density, indicating the existence of a limited number of "very leaky" vesicles within the total vesicle population in the incubation mixture. For a given set of experimental conditions (vesicle lipid concentration, -composition, temp., etc.) the initial rate at which these "very leaky" vesicles release their contents apparently increases linearly with the number of cells in the incubation. Taking into consideration again, that for either cell type $\Delta\Lambda$ always reaches a maximum (after approximately 10 min of incubation time), we conclude that: first, a limited fraction of the total vesicle population is subject to induced leakage, and second, the size of this fraction is a function of cell-density and reaches a maximum at a cell-density > 10^7 cells/ml.

Influence of vesicle lipid concentration

At a fixed cell-density, $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ also depend on the vesicle lipid concentration, as is depicted in Fig. 3. $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ have maximal values at vesicle lipid concentrations of 0.0014 mM and 0.0028 mM, respectively. With increasing vesicle lipid concentration $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ are found to decrease. At vesicle lipid concentrations below 0.0014 mM and 0.0028 mM the values of the leakage parameters are consistently found to be submaximal (dashed lines) but although their determination is sufficiently reliable, considerable inter-experimental variation is seen. The fraction of vesicles which is found cell-associated (after 10 min of incubation time) depends on vesicle lipid concentration in much the same way as $\Delta\Lambda_{max}$ (Fig. 3, crosses and filled circles, respectively). This similarity suggests that the number of vesicles subject to induced leakage is correlated to the number of vesicles bound by the cells. Quantitatively, the fraction of cell-associated vesicles is approx. 5 times smaller than the fraction which becomes leaky. It has to be kept in mind, however, that in order to assess the amount of cell-associated vesicle-lipid, bulk (*i.e.* non cell-associated) vesicles have to be removed by several washings, possibly causing loosely cell-associated vesicles to detach from the vesicle-cell complex, resulting in an underestimation of the amount of cell-associated vesicles. During a 10 min incubation any mechanical disturbance of

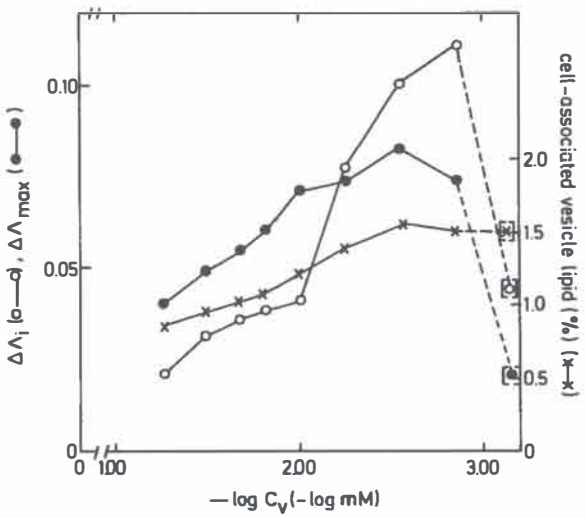

Fig. 3. *Cell-induced leakage of carboxyfluorescein from vesicles as a function of vesicle lipid concentration.* 10^7 Zajdela cells in Hanks'BSS were added to different final concentrations of 100 mM carboxyfluorescein containing vesicles, consisting of [N-methyl[^{3}H]] egg PC, cholesterol, and PS (molar ratio 5 : 4 : 1), in a final volume of 3 ml. For each vesicle lipid concentration used, fluorescence development was followed as described in the legend of Fig. 1 and in the text; the resulting $\Delta\Lambda$ values ($\Delta\Lambda_i$ O——O; $\Delta\Lambda_{max}$ ●——●; for symbols see legend to Fig. 2) were plotted against the vesicle lipid concentrations. After $\Delta\Lambda_{max}$ had been attained (10 min) the incubation mixtures were transferred to polystyrene tubes. Non cell-associated vesicles were removed by repeated centrifugations and washings (NaCl/Hepes buffer pH 7.4). The washed cells were then assayed (see materials and methods) for cell-associated [^{3}H]labeled PC radioactivity (X——X). C_v: vesicle lipid concentration (mM).

the incubation mixture in the cuvet (*e.g.* gentle stirring with a plastic rod) results in a small superimposed leakage jump (superimposed B-type curve; see Fig. 1). Since much manipulations do not result in an increase of the monitored fluorescence output, F_d, if only vesicles or cells are present, we tend to conclude that slight mechanical disturbance lead to a rearrangement of vesicles and cells within the system: presumably a minor fraction of the cell-associated "very leaky" vesicles easily dissociate from their cell surface binding sites, back into the medium, and are replaced by bulk vesicles which - in turn - become subject to leakage induction.

The most obvious explanation for the observation that $\Delta\Lambda_{max}$ reaches to a

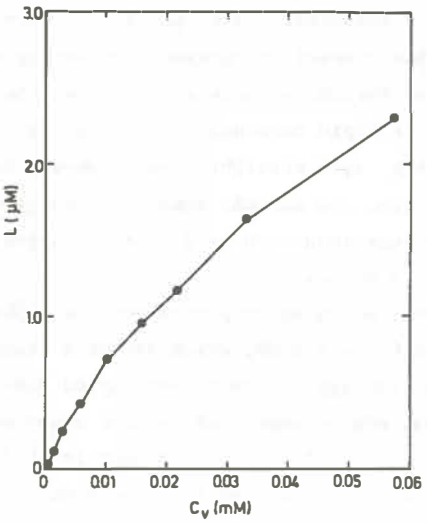

Fig. 4. *Absolute cell-induced leakage of carboxyfluorescein from vesicles as a function of vesicle lipid concentration.* Values in this Figure were calculated from Fig. 3 by multiplying, for each vesicle lipid concentration, the values of $\Delta\Lambda_{max}$ with the corresponding vesicle lipid concentration, yielding the amount of vesicles (L; expressed as nmoles vesicle lipid per ml) that could be considered to have completely released the entrapped dye. C_v: vesicle lipid concentration.

maximal value is given on the assumption of an average limited leakage-inducing capacity per cell. At the minimal vesicle concentration required to saturate the leakage inducing capacity, a maximal fraction of vesicles releases its content. Both at higher as well as lower vesicle concentrations a smaller fraction of vesicles would then be involved in cell-induced leakage: *i.e.* $\Delta\Lambda_{max}$ would then be lower. At the lowest vesicle concentration however, this fraction is obviously not related to total cell-associated lipid. At present we cannot explain this discrepancy. Apparently, the initial rate of induced leakage reaches a maximum at a lower vesicle concentration than $\Delta\Lambda_{max}$. Although we have no clear explanation for this difference in optimal (for leak induction) concentration, it could be suggested that at vesicle lipid concentrations > 0.0014 mM the leak-inducing "sites" become progressively less accessible to other vesicles binding to the cells surface, while at the same time the degree of saturation of the remaining (fully exposed) leak-inducing sites increases

i.e. at least up to a vesicle lipid concentration of approximately 0.0028 mM.

If we calculate the absolute amount of vesicles (expressed as nmol vesicle lipid/ml, subject to induced leakage) that completely releases the entrapped dy, as a function of vesicle lipid concentration, a curve (Fig. 4) is obtained which tends to saturate at high vesicle lipid concentration; on double reciprocal plotting of the parameters of Fig. 4, a straight line is obtained. For an infinite vesicle lipid concentration, the maximal number of vesicles (expressed as vesicle lipid) subject to leak induction by 3.33×10^6 Zajdela cells (10 min) can then be calculated: 4.5 nmoles.

The results described above reveal that at relatively high vesicle lipid concentrations (*e.g.* within the range of 0.1 - 1.0 mM, which has been studied most extensively in many investigations) leakage of vesicle-entrapped substances due to vesicle-cell interactions, may go unnoticed, as the assay methods usually average the solute release from all vesicles present in an *in vitro* incubation with cells. For vesicles at the cell surface, however, the magnitude of the leakage induction phenomenon is not negligible (*cf.* Van Renswoude *et al.*, 1979).

In order to show that the phenomena observed are not merely associated with the use of carboxyfluorescein as an entrapped compound, we also encapsulated HRPO and FITC-dextran and measured parameters of leakage.

Influence of species and concentration of entrapped substance

If, in a 10 min incubation with cells, instead of carboxyfluorescein, horse radish peroxidase (M.W. 32 000) is used as a vesicle-entrapped marker (Fig. 5), the latency of the enzyme inside vesicles appears to decrease with decreasing vesicle concentration in much the same way as the $\Delta\Lambda_{max}$ is found to increase when using carboxyfluorescein-containing vesicles (*cf.* Fig. 3). Unfortunately, the sensitivity of the assay does not allow examination of the latency dependence at very low vesicle concentrations. With FITC-labeled dextran (average M.W. 3 000) as an encapsulated marker, a fluorescence jump similar to that observed with carboxyfluorescein is recorded upon addition of cells (results not shown).

Vesicles carrying 200 mM carboxyfluorescein yield about three-fold higher $\Delta\Lambda_{max}$ values than vesicles containing 100 mM dye. Presumably the 200 mM vesicles are less stable than the 100 mM vesicles, this lower stability partially being expressed at the level of vesicle-cell surface interaction. The higher

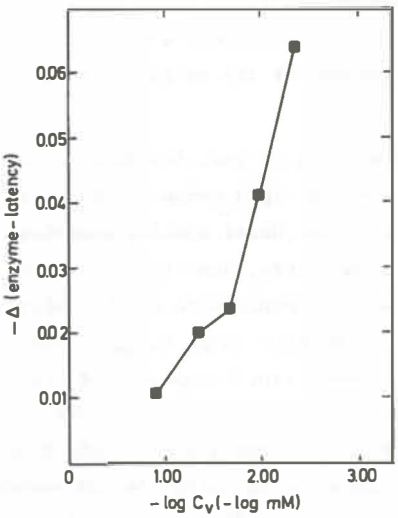

Fig. 5. *Cell-induced leakage of horse radish peroxidase from vesicles as a function of vesicle lipid concentration.* 10^7 Zajdela cells in Hanks'BSS were added to different final lipid concentrations of vesicles, consisting of PC/cholesterol/PS (molar ratio 5 : 4 : 1) and containing horse radish peroxidase at a concentration of 50 mg/ml. Final volume: 3 ml. Leakage of the enzyme (expressed as -Δ latency; see below) was assessed after 10 min of incubation time. For description of the assay of enzyme-activity and incubation conditions see Materials and Methods. Latency is defined as 1 -(activity in absence of detergent divided by activity in presence of detergent). The presence of detergent, Triton X-100, final conc. 0.1% (v/v), did not affect the enzyme activity. Within 10 min of incubation time cells did not release peroxidase-activity into the medium. Values of -Δ (enzyme-latency) were corrected for bulk leakage (*i.e.* in absence of cells) of the enzyme from vesicles at different vesicle concentrations.

$\Delta\Lambda_{max}$ values obtained with 200 mM dye containing vesicles also practically rule out the possibility that an increase of Λ is caused by an increase of the internal aqueous volume of the vesicle since there is no significant difference in the degree of self-quenching of 100 or 200 mM dye inside vesicles (*cf.* Van Renswoude *et al.*, 1979).

From the results described so far we tentatively conclude that, in the presence of either isolated hepatocytes or Zajdela cells, small unilamellar vesicles composed of PC, cholesterol and PS (molar ratio 5 : 4 : 1) show a rapid loss of integrity, expressed by the release of their contents into the

medium; this phenomenon seems to be confined to a minor fraction of the total vesicle population. The magnitude of the leakage induction seems to be correlated to the vesicle fraction that is found cell-associated, and not to depend critically on the type of entrapped substance or its molecular weight.

Influence of vesicle lipid composition

As there is a large variety in lipid composition of vesicles used by different investigators we tested vesicles of various lipid compositions with regard to their integrity in the presence of cells. Under similar experimental conditions (with respect to incubation temperature, vesicle lipid concentration and cell density) as used for the experiments with PC/cholesterol/PS (molar ratio 5 : 4 : 1), no significant fluorescence jumps ($\Delta\Lambda_{max}$ values < 0.01) were observed with vesicles of the following lipid compositions: PC alone; PC/cholesterol, with cholesterol contents up to 30 mole %; PC/PS with a PS-content up to 10 mole %; and PC/cholesterol/PS comprising 10 mole % of PS and up to 20 mole % of cholesterol. Upon addition of cells, PC/cholesterol mixtures containing more than 30 mole % of cholesterol display a distinct fluorescence jump ($\Delta\Lambda_{max}$ > 0.06) which steeply increases with increasing mole fraction of cholesterol (Fig. 6). This is a rather paradoxical result, since bulk leakage (*i.e.* in the absence of cells) of carboxyfluorescein from vesicles at the same time is found to decrease slightly with a cholesterol-content increasing from 30-40 mole %. We furthermore observed that the presence of 10 mole % of net negatively charged lipid species (PS or DCP) in PC/cholesterol mixtures leads to a significant leakage induction ($\Delta\Lambda_{max}$ > 0.06) even at cholesterol contents exceeding 20 mole %. Our results suggest, therefore, that the vesicle cholesterol plays an important role in the observed leak induction by cells, especially when the vesicle membrane simultaneously contains minor amounts of negatively charged lipid.

Preliminary results indicate that the phenomenon is not restricted to sonicated small unilamellar vesicles: reverse phase evaporation vesicles (Szoka *et al.*, 1978) made up from lipid compositions which for small unilamellar vesicles lead to significant Δf_{max} values, show similar behaviour in the presence of cells. All highly sonicated vesicle preparations used in this study were devoid of significant contamination with multilamellar liposomes, as judged by Sepharose CL-4B chromatography: on the average, more than 98% of lipid phosphorus elutes in the

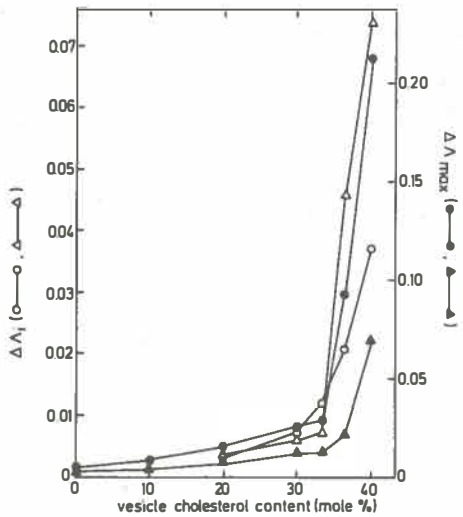

Fig. 6. *Influence of vesicle-cholesterol content on cell-induced leakage.* Vesicles consisting of PC and cholesterol in different molar ratios were incubated with either 2 x 10^6 hepatocytes (triangles) or 10 Zajdela cells (circles). Release of carboxyfluorescein from vesicles was monitored and evaluated as described in the legends of Figs. 1 and 2. The initial rate of cell-induced leakage ($\Delta\Lambda_i$): open symbols, maximal induced leakage ($\Delta\Lambda_{max}$): filled symbols. For further details see Materials and Methods.

included volume. Centrifugation (100 000 x g; 1 hr) of sonicated preparations does not result in a change of the values of the leakage parameters. Thus, a possible small contamination of the preparations with multilamellar vesicles cannot per se be responsible for the occurrence of induced leakage.

Tentative characterization of leak-inducing activity, with isolated plasma membranes

The time-dependent kinetics of dye-release observed with intact cells are similar to those seen with isolated plasma membranes (*cf.* Fig. 1): *i.e.* a rapid fluorescence jump which levels off to a plateau value within approx. 10 min. Again, as was already discussed for intact cells, the results suggest the existence of a fraction of vesicles subject to leakage induction. The likelihood of the existence of such a fraction is substantiated by the finding

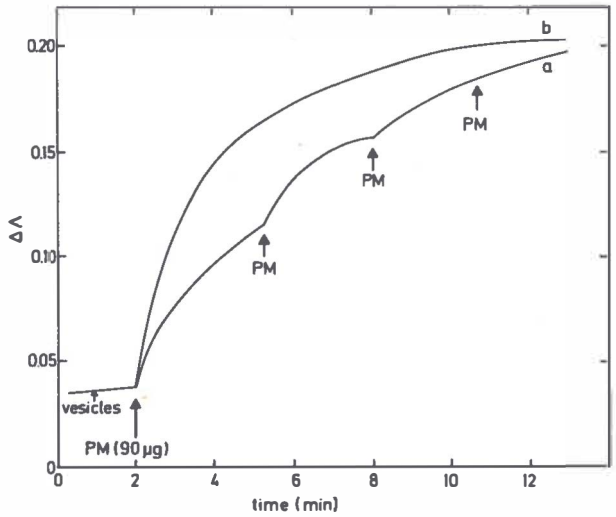

Fig. 7. *Saturability of plasma-membrane induced leakage upon sequential addition of plasma membranes.* To 0.01 mM (final lipid concentration) vesicles, consisting of PC/cholesterol/PS (molar ratio 5 : 4 : 1), 90 µg (protein weight) of hepatocyte plasma membrane preparation was added at t=2 min (arrow). Equal portions were then added sequentially at t = 5, 8 en 11 min (arrows, curve a). The volume change of the system amounted to approx. 0.33% per addition. Carboxyfluorescein release was monitored and numerically evaluated as described in legends of Figs. 1 and 2. Curve B represents the induced leakage of dye upon one single addition of 360 µg of plasma-membranes to 0.01 mM vesicles.

(Fig. 7) that sequential additions of small aliquots of plasma membranes (Fig. 7, curve a) lead to the same final $\Delta\Lambda_{max}$ value as is found when the sum of these aliquots is added at once (Fig. 7, curve b). The leak induction capacity of a fixed amount of plasma membranes furthermore appears to be limited: upon sequential additions of aliquots of dye-containing vesicles, the final $\Delta\Lambda_{max}$ value is roughly equal to the corresponding value resulting from addition of all aliquots at once (data not shown). Also, leakage induction is found to depend on vesicle lipid concentration in the same way as is observed for whole cells. For a given vesicle concentration, however, the value of $\Delta\Lambda_{max}$ generally is slightly higher than the one observed with cells. It was assumed (Ray, 1970) that plasma protein accounts for approx. 3% of total cellular proteins. We used this value to calculate the amount of plasma membrane equivalent to a certain number of cells.

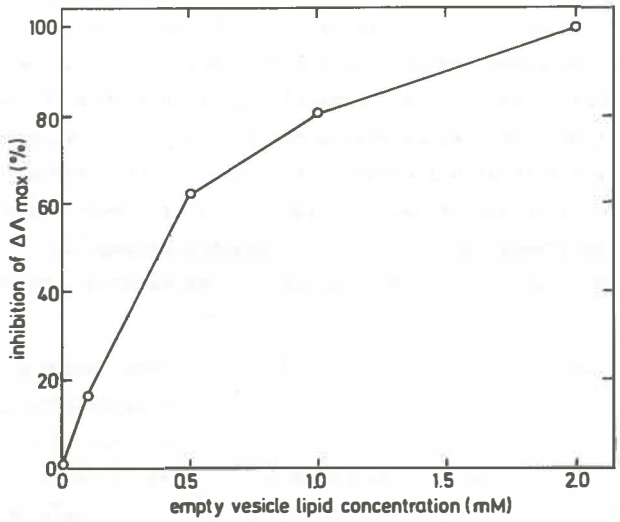

Fig. 8. *Inhibition of plasma membrane induced leakage by empty vesicles.* 90 µg (protein weight) of hepatocyte plasma membranes were incubated with empty vesicles [(PC/cholesterol/PS (5:4:1)] during 5 min at 37 °C in NaCl/Hepes buffer, pH = 7.4. Then carboxyfluorescein-containing vesicles of the same lipid composition were added (final concentration : 0.01 mM), and $\Delta\Lambda_{max}$ was determined (see legends of Figs. 1 and 2).

Addition of carboxyfluorescein-vesicles to plasma membranes which were premixed (during 5 min at 37 °C) with increasing amounts of empty vesicles results in an almost complete abolishment of leak induction at high empty vesicle concentration (Fig. 8). This result suggests, that empty vesicles compete with dye-containing vesicles for the "leakage inducing sites" and that these sites only become saturated at relatively high overall vesicle lipid concentration.

Influence of various treatments on leak induction

No significant loss of leakage-inducing capacity of plasma membranes is seen when they are preheated to 100 °C (boiling water bath) during 30 min. Hence we consider it very unlikely that plasma membrane intrinsic enzymic activity (*e.g.* phospholipases) accounts for the induced leakage. Treatment of plasma membranes with trypsin (15 µg of enzyme per 90 µg protein weight of plasma membranes; incubation volume 3 ml; 10 min; 37 °C; reaction stopped by

addition of 35 µg of trypsin inhibitor) decreased $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ 4- and 7-fold, respectively. Upon neuraminidase treatment (9 µg of enzyme per 90 µg protein weight of plasma membranes; incubation volume 3 ml; 30 min; 37 °C), $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ were reduced 3- and 4-fold, respectively. When either enzyme was added to plasma membranes immediately before addition of vesicles, or when vesicles were premixed with either enzyme, the values of the leakage parameters were unaltered. With intact cells neuraminidase treatment had a similar effect. From these results we conclude that induced leakage is mediated through one or more cell-coat- or intrinsic plasma membrane proteins, most likely glycoprotein(s).

Release of leak inducing activity from cells and isolated plasma membranes

Upon prolonged (up to 3 hrs) incubation of cells or plasma membranes, at 37 °C in Hanks' Basal Salt Solution, we detected a gradual appearance of leak-inducing activity in the medium itself (assessed after removal of cells or plasma membranes by centrifugation), at the cost of the cell- or plasma membrane-associated activity (assessed after pelleting and resuspending the cells or membranes). After 1 hr cells and plasma membranes gave 80% and 65% of their initial (zero-time) $\Delta\Lambda_{max}$ values, respectively. With PC/cholesterol/PS (5:4:1) vesicles the medium itself then displayed low leakage-inducing activity ($\Delta\Lambda_{max} < 0.02$) with kinetic characteristics (cf. Fig. 1) similar to those of cell- and plasma membrane-induced leakage. Repeated freezing and thawing of isolated plasma membranes leads to loss of almost all membrane-associated activity, while at the same time the medium gives rise to $\Delta\Lambda_{max}$ values > 0.06 (assessed after removal of plasma membranes by centrifugation). Such medium was found to contain no detectable protein using the protein determination method according to Lowry (1951), and was therefore incubated with carrier-free ^{125}I according to Bocci (1969). After dialysis overnight, the medium was subsequently chromatographed on Sepharose CL-6B (Fig. 9). Whilst ^{125}I-labelled material eluted throughout the column volume (fractions 5 to 26), leak-inducing activity was found only in fractions 14 to 19, indicating that, at that stage, it was no longer associated with membraneous structures, which would elute in the void volume. The leak-inducing activity in fractions 14 to 19 was almost completely abolished upon treatment with either trypsin or neuraminidase, according to the protocol described above. From the results obtained so far we tentatively conclude that most likely the leakage-inducing factor

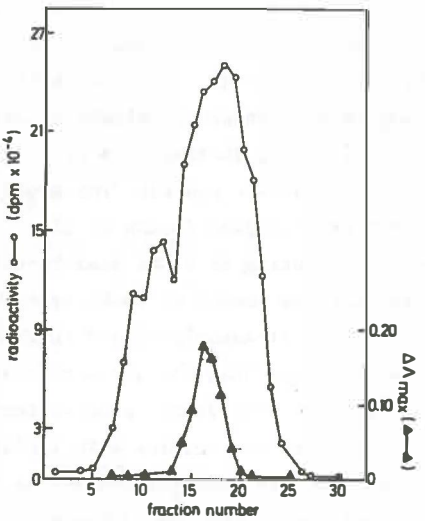

Fig. 9. *Gel chromatographic elution profile of leakage inducing activity.* Hepatocyte plasma membranes (suspended in NaCl/Hepes buffer, pH 7.4) were put through four freezing and thawing cycles, and subsequently centrifuged at 100 000 xg for 30 min (Spinco L2-65 B, Ti50 rotor). The supernatant was adjusted to pH 8.0 by addition of 0.15 M citrate. 500 µCi of carrier free ^{125}I (The Radiochemical Center, Amersham) was then added to the supernatant, in order to label peptides and proteins according to Bocci (1969). After a reaction-time of 30 min, the mixture was dialyzed (overnight), against 3 changes of a 300-fold excess of NaCl/Hepes buffer pH 7.4, to remove non-bound ^{125}I-label and labeled materials with a M.W. < 10000. Subsequently, the dialyzed supernatant was chromatographed on Sepharose CL-6B (column 1.5 x 50 cm, equilibrated and eluted with NaCl/Hepes buffer pH 7.4). 1 ml fractions were collected; radioactivity (0——0) and leak-inducing activity ($\Delta\Lambda_{max}$; ▲——▲) were determined for each fraction. For measurement of leak induction, 0.01 mM PC/cholesterol/PS (5:4:1) vesicles, containing 100 mM carboxyfluorescein, were used.

consists of one or more glycoproteins, and that it is rather loosely associated with the cell-surface.

Concluding remarks

Attempts to give a general explanation for our observations meet with two important questions:

(i) which factor does - ultimately - initiate leakage?

(ii) is any vesicle within the total vesicle population prone to induced leakage or is this phenomenon restricted to a subpopulation of vesicles?

Possibly, leakage starts upon interaction of cell-coat protein(s) with the vesicle bilayer. There is a great deal of evidence that vesicles of different lipid compositions readily associate with exogenous proteins (see the review by Tyrrell *et al.*, 1976; Kimelberg, 1976; Zborowski *et al.*, 1977; Hoekstra *et al.*, 1979) and that such association may lead to enhanced release of solutes from the vesicle interior (Tyrrell *et al*, 1976; Zborowski *et al.*, 1977). More specifically, it has been shown, that cell-surface protein from mouse spleen cells (Dunnick *et al.*, 1976) and from erythrocytes (Bouma *et al.*, 1977) is partly transferable to (bulk) vesicles during *in vitro* vesicle-cell incubations. In this report we demonstrate that the number of leaky vesicles is proportional to the number of vesicles found cell-associated and that vesicles are subject to cell-induced leakage only when they contain more than 30 mole % of cholesterol or more than 20 mole % of cholesterol plus at least 10 mole % of negatively charged lipid. Furthermore, our results with isolated plasma membranes indicate a direct involvement of cell-surface protein in the process of leak induction. We consider it conceivable that an isothermal phase separation within the vesicle membrane ultimately leads to the observed leakage. Such a phase separation could either be induced *in situ* upon interaction with cell-surface proteins (*cf.* Papahadjopoulos, 1977) or preexist (*i.e.* present before the vesicle makes intermolecular contact with the cell-surface) and thus highly facilitate the interaction of the vesicle membrane with proteins, in much the same way as - for instance - the attack of liposomes by high density lipoproteins (Scherphof *et al.*, 1979) or by phospholipase A_2 (Wilschut *et al.*, 1979). A (preexisting) phase separation was also suggested to facilitate the interaction of cell-surface proteins with vesicles consisting of dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine (molar ratio 1 : 1) which were found highly leaky in the presence of cells (Szoka *et al.*, 1979). A preexistent isothermal phase separation could result from the influence of non-specific interactive forces [long-range Van der Waals attractive-, electrostatic- and strong repulsive "hydration" forces (Parsegian *et al.*, 1979)] upon the vesile bilayer approaching the negatively charged cell surface (*cf.* Sugár, 1979). Alternatively, it could be an intrinsic property of the vesicle, determined by its lipid composition (*cf.* Gebhardt *et al.*, 1976). The physical properties of vesicles made up from egg PC and cholesterol change drastically as soon as the overall cholesterol-content starts to exceed 30 mole %; there is a marked increase of average size as

well as a considerable alteration of transbilayer distribution of the constituent lipids (De Kruijff *et al.*, 1976; Forge *et al.*, 1978; Carnie *et al.*, 1979). Furthermore, the degree of intermolecular interaction between the phospholipid polar head-groups will be highly restricted: instead of being predominantly oriented in the plane of the vesicle bilayer (PC alone or PC plus low amounts of cholesterol) the head-groups will be extended out from the bilayer surface (Yeagle *et al.*, 1977). If vesicles consist of PC, cholesterol and PS, the latter known to display a much higher affinity towards cholesterol than PC (Van Dijck, 1979), the possibility of an intrinsic- or induced isothermal phase separation is even more likely. The existence of subpopulations within a vesicle population made from binary- or ternary lipid mixtures cannot be ruled out: for instance, a combination of the data from the studies of Forge *et al.* (1978) and De Kruijff *et al.* (1976) suggests that a vesicle population made of PC and more than 30 mole % of cholesterol must be inhomogenous, either with respect to the transbilayer distribution of lipids per individual vesicle or to the molar ratio of lipids per individual vesicle. Our results with reverse phase evaporation vesicles argue against and important role of size (per se) of the vesicles in their susceptibility to leak induction.

Although at present we are unable to indicate which (combination) of the considerations displayed above would be the one of choice to apply to our data, we consider the results of this study important from a phenomenological as well as from a methodological point of view, for three main reasons. Firstly, the observed leakage phenomenon may hamper the identification of different classes of vesicle-cell interaction sites (Blumenthal *et al.*, 1977; Van Renswoude *et al.*, 1979; Hoekstra *et al.*, submitted). Transfer of carboxyfluorescein from vesicles to cells seems not to be affected by the occurrence of induced leakage: vesicles composed of PC/cholesterol/PS in a molar ratio 5:4:1 or 7:2:1 (the latter composition not being subject to induced leakage) display identical initial transfer-rates. Also, pretreatment of hepatocytes with neuraminidase does not result in a change in transfer-rate, whereas the amount of cell-associated, highly self-quenched vesicle-carboxyfluorescein is found to be increased (Hoekstra, unpublished data). Secondly, use of the described test system may facilitate the choice of a certain vesicle lipid composition to be studied in vesicle-cell interactions. Thirdly, our methodological approach may constitute a versatile basis for further exploration of some of

the characteristics of (complex) vesicle-cell interaction, which, in turn, could lead to a better understanding of cell-surface properties, like localized heterogeneity, local surface curvature, transbilayer asymmetry, and reactivity of the cell-coat.

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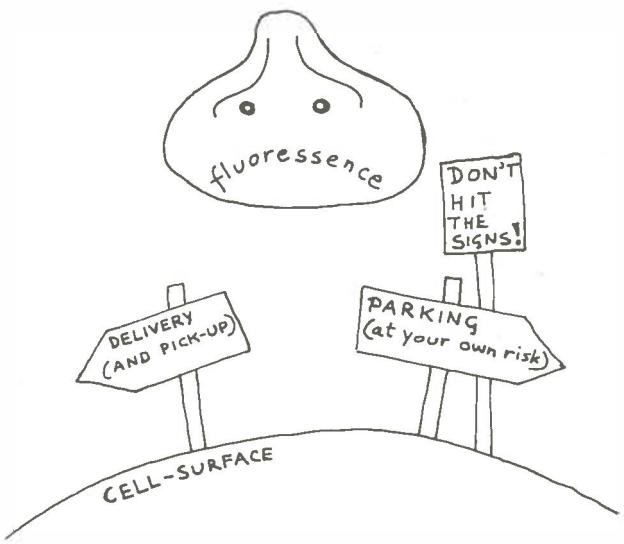

A.J.B.M. van Renswoude and D. Hoekstra

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CHAPTER V

ASPECTS OF LIPOSOMES AS *IN VIVO* DRUG-CARRIERS: EFFECT OF ENCAPSULATION OF 1-β-D-ARABINOFURANOSYL-CYTOSINE WITHIN LIPOSOMES ON ITS EFFICACY AGAINST MOUSE SPLEEN LYMPHOSARCOMA

SUMMARY

This Chapter describes a pilot study on the effect of encapsulation of 1-β-D-arabinofuranosyl-cytosine within liposomes on its activity, *in vivo*, against the growth of a murine spleen lymphosarcoma. Multilamellar liposomes consisting of phosphatidylcholine, cholesterol and phosphatidylserine, in a molar ratio of 5 : 4 : 1 or 7 : 2 : 1, are used. Upon intraperitoneal administration, the drug is found to inhibit DNA-synthesis in the tumor more effectively when given encapsulated in liposomes than in free form. In this respect, liposomes which contain 20 mole % of cholesterol are found to be superior to those containing 40 mole % of cholesterol. *In vitro* leakage, both in plain buffer as well as in mouse blood, of the drug from the liposomes is shown to be inversely correlated to the liposome-cholesterol content. Use of fluorescein-isothiocyanate tagged dextran and radio-iodinated polyvinylpyrrolidone indicates that liposomes can leave the peritoneal cavity and most likely reach the spleen tumor via the lymphatics and blood incubation. We tentatively conclude that the liposomes act as a moving depot from which the drug is released steadily. The rate of this release will, inter alia, depend on the liposome-cholesterol content. At present we cannot indicate whether and to what extent the drug might be delivered directly into the target cells, via liposome-cell contact.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PC) and cholesterol (CH-S-grade) were obtained from Sigma. Phosphatidylserine (PS) was isolated from bovine brain extract (Sigma) by preparative silica-gel thin-layer chromatography. [*Me*-^{14}C] phosphatidylcholine (specific activity 2.2 Ci/mole) was prepared from egg yolk PC essentially as described by Stoffel *et al.* (1). All lipids used gave

one spot on silica-gel thin-layer chromatograms, with $CHCl_3/CH_3OH/H_2O$ (65 : 35 : 4, by volume) as an eluant. HEPES and 1-β-D-arabinofuranosylcytosine (Ara-C) were from Sigma; FITC-dextran (average M.W. 3000) was purchased from Pharmacia. [^{125}I]-labeled polyvinylpyrrolidone (PVP) (specific activity 46.3 μCi/mg, average M.W. 35000), [6-^{3}H]Thymidine (5 Ci/mmole) and [5-^{3}H]Ara-C (14.7 Ci/mmole) were bought from The Radiochemical Centre, Amersham, England.

Cells

Lymphosarcoma spleen cells were transplanted weekly, as a routine, in C57 BL mice (strain: Laboratory of Radiopathology, State University of Groningen, The Netherlands) by intraperitoneal injection of 10^6 cells in 0.5 ml of sterile saline, as described by Konings and Rasker (2). *In vivo* experiments were carried out on the seventh day after inplantation of the tumor. Lymphosarcoma spleens weighed then about 800 mg. Imprints, stained with May-Grünwald-Giemsa, of such spleens showed almost exclusively lymfosarcoma cells. For isolation of tumor cells see the legend to Table 1. DNA-synthesis in spleens was quantitated by measuring the incorporation of [^{3}H]Thymidine, as described by Konings and Trieling (3).

Liposomes

Chloroform solutions of PC, cholesterol, and PS were mixed in a molar ratio of 5 : 4 : 1 or 7 : 2 : 1 and evaporated in vacuo or under a stream of nitrogen. The dry lipids were vortex-dispersed in an appropriate buffer containing Ara-C, radiotracers or a fluorescent marker (for buffers and concentrations of entrapped substances see the legends to Figures and Tables). The final lipid concentration in the dispersions was usually 25 mM. Subsequently, the dispersions were sonicated, under flushing with nitrogen, for 15 s to break up very large lipid aggregates (Branson B15 sonifier, titanium microprobe, nominal energy output 40 W). The preparations were then centrifuged at 2000 x g for 10 min. The pellet, containing (on the average) 30% of the total lipid weight, was discarded. The size of the liposomes in the supernatant was estimated by Sepharose 4B column chromatography, or, at a later stage of the study, with a - more convenient - thin-layer gel-chromatographic method (4). More than 95% of the lipid was found to elute in the void volume. For routine use, liposomes were freed from non-entrapped substance by column chromatography with Sepharose G-100 (for Ara-C-containing vesicles) or with Sepharose

6B (for FITC-dextran- or [^{125}I]PVP-containing liposomes). On the average, the liposomes entrapped 0.8 µl of aqueous volume per µmole of total lipid (based upon [^{3}H]Ara-C). Leakage of [^{3}H]Ara-C from liposomes was determined by equilibrium dialysis with a Dianorm^{R} apparatus (Innovativ Medizin Ltd, Zürich, Switzerland) using teflon cells in which two 1-ml compartments were separated by a Spectrapor II dialysis membrane. Fluorescence measurements were carried out and processed as described elsewhere (5). ^{125}I-radioactivity was determined with a Searl γ-counter (efficiency approx. 80%). Radiocarbon- and tritium radioactivity were assessed with a Nuclear Chicago MkII liquid scintillation counter, using a xylene-based scintillation mixture (6).

INTRODUCTION

There is a growing consensus of opinion that liposomes* are potentially promising as *in vivo* vehicles for antineoplastic agents (see, for example, Refs. 7-9). As such, liposomes meet some important demands regarding ideal carrier-properties: made up from naturally occurring substances, they are biodegradable and non-toxic; their physico-chemical properties like size, charge and composition can be manipulated to a considerable extent without affecting their basic appearance: an aqueous compartment surrounded by a lipid bilayer (*cf.* Chapter I).

Virtually all drugs which are used in the treatment of cancer exhibit a great deal of undesired side-effects, such as bone-marrow depression, debilitation of the immune defence-system, gastro-intestinal disorders, and damage to the reproductive system. The severity of these side-effects often depends on the dose of the drugs and thus may limit a full use of their antineoplastic potential.

The pyrimidine analogue 1-β-D-arabinofuranosyl-cytosine (Ara-C), whose potent antitumor effect is based on inhibition of DNA-synthesis (10-13) is used in many chemotherapeutic regimes against leukemia (14,15). In 1975 Kobayashi and colleagues (16) reported that entrapment of Ara-C in positively charged multilamellar liposomes led to a dramatic enhancement of its *in vivo* efficacy against the murine leukemia L1210. Similar results were obtained by Mayhew *et al.* (17): a single intraperitoneal dose of Ara-C, encapsulated in small unilamellar, negatively charged vesicles, effectuated a doubling of the

* In this report the terms "liposome" and "vesicle" will be used interchangeably.

average survival time of L1210-bearing mice, whereas Ara-C, administered as free drug, had no effect under such circumstances. Although - obviously - an increase of survival time should be considered an important - if not ultimate - goal to aim at in cancer chemotherapy, we preferred to measure the efficacy of Ara-C against an experimental tumor in mice in a more direct way: be determining the rate of DNA-synthesis in the tumor cells. Since DNA-synthesis in murine spleen lymphosarcoma (for details on this tumor see Refs. 2,3,18) was reported by Konings and Trieling (3) to be inhibited by intraperitoneally administered Ara-C, we chose this tumor as a first experimental model system to investigate the application of multilamellar liposomes as *in vivo* carriers for Ara-C. The results of this pilot-study are presented here.

RESULTS

Upon a single intraperitoneal injection of liposome-entrapped Ara-C or free Ara-C plus empty liposomes, DNA-synthesis in spleen lymphosarcoma cells is found to be inhibited, as is shown in Fig. 1. Until about 100 min after injection the three different preparations are found te be roughly equally effective. Thereafter, DNA-synthesis in the tumor starts to recover: relatively quickly when free Ara-C had been administered, and significantly slower when the drug was given encapsulated within liposomes. Apparently, the liposomes with the lower cholesterol content are the most effective in keeping DNA-synthesis at a reduced level. At 250 min after the injection of drug-containing liposomes which comprise 20 mole % of cholesterol, DNA synthesis is found to be inhibited by about 60%. With liposomes containing 40 mole % cholesterol the degree of inhibition amounts to approximately 35%. The rationale for using liposomes with different cholesterol-contents was provided by the presumption, based upon the work of others (19,20), that Ara-C would leak at different rates from liposomes with different cholesterol-contents. In view of the apparent difference (Fig. 1) in antitumor effect between liposome preparations with different mole fractions of cholesterol, we subjected [^{3}H] Ara-C-containing liposomes of either lipid composition to equilibrium dialysis against liposome-free medium, in order to determine leakage of the drug from the vesicles. To mimic *in vivo* conditions we measured leakage both in buffer as well as in heparinized mouse blood, thus avoiding the experimentally very

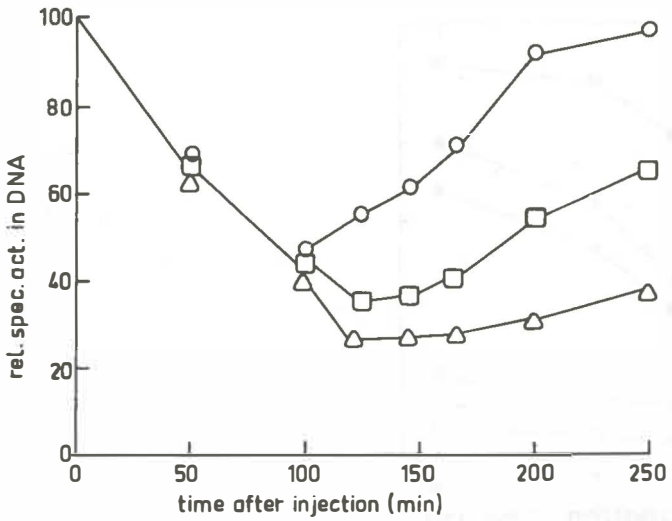

Fig. 1. In vivo *inhibition of DNA-synthesis in spleen lymphosarcoma cells by free and liposome-encapsulated Ara-C.* Lymphosarcoma-bearing mice received an intraperitoneal injection of 0.5 ml 0.125 M NaCl/0.050 M Tris (pH 7.4) containing 0.275 mg Ara-C (corresponding to 13.7 mg/kg body weight), either as free drug, or entrapped in 2.1 µmoles of liposomal lipid. The multilamellar liposomes consisted of PC, cholesterol, and PS (molar ratios 5 : 4 : 1 or 7 : 2 : 1). In order to assess the degree of inhibition of DNA-synthesis at time t (abscissa) after drug administration, 10 µCi of [^{3}H]Thymidine (s.a. 5 Ci/mmole) in 250 µl of sterile saline was injected intraperitoneally at t minus 15 min. At time t, the animals were sacrified, their spleens removed, and the specific activity of [^{3}H]Thymidine in the DNA of spleen lymphosarcoma cells was determined (see Materials and Methods). The measured specific activities are given as percentages (ordinate) of values obtained from untreated control animals (usually about 50 dpm/µg DNA). Each point represents the mean of values of four separate experiments differing not more than 15%. Circles: free Ara-C. Squares: Ara-C entrapped in vesicles with 40 mole % of cholesterol. Triangles: Ara-C entrapped in vesicles with 20 mole % of cholesterol.

difficult approach of measuring leakage after actual injection in the peritoneal cavity. The results are given in Fig. 2. In buffer, liposomes comprising 40 mole % of cholesterol release the entrapped drug much more slowly than liposomes with 20 mole % of cholesterol. In mouse blood this difference is far less pronounced, while at the same time the rate of leakage of the drug from either type of vesicles is found to be drastically increased. Similar results concerning a leakage-enhancing effect of blood were reported by

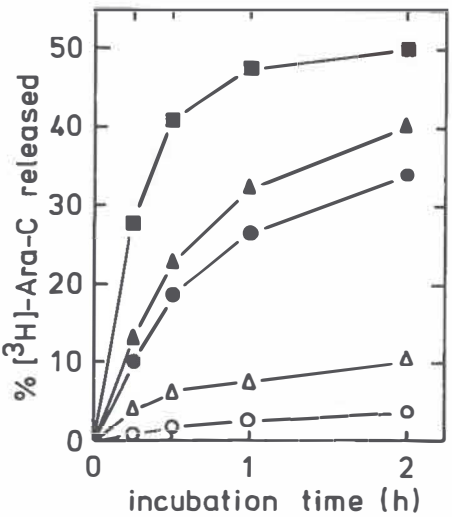

Fig. 2. *Leakage of Ara-C from multilamellar vesicles* in vitro. Leakage of the drug from liposomes was determined using an equilibrium dialysis set-up (see Materials and Methods). Multilamellar liposomes consisted of PC/cholesterol/PS (molar ratio 5 : 4 : 1 or 7 : 2 : 1) and carried 0.1 µCi of entrapped [^{3}H] Ara-C (final specific activity 0.63 µCi/mg) per µmole of total lipid. Liposome-preparations were present in the left compartments of the dialysis cells; both halves of each cell contained either 0.125 M NaCl/0.050 M Tris (pH 7.4) or heparinized mouse blood, mixed with NaCl/Tris buffer in a ratio of 7 : 1 (v/v). The final incubation volume in each cell-half was 1 ml; the final lipid concentration in the left halves was 1.6 mM. Dialysis was carried out at 37 °C in a thermostatted water bath. At suitable times (abscissa) 50 µl samples were taken from each cell-half and assessed for radioactivity. The percentage of released [^{3}H]Ara-C (ordinate) is calculated as $[X_R/(X_R+X_L)]\times 100\%$, where X_R and X_L represent the measured radioactivities in samples from the right- an left cell-half, respectively. Filled symbols: leakage in presence of blood. Open symbols: leakage in NaCl/Tris. Triangles: PC/cholesterol/PS, molar ratio 7 : 2 : 1. Circles: PC/cholesterol/PS, molar ratio 5 : 4 : 1. Filled squares: control (free Ara-C). Each point represents the average of the values of a duplicate experiment agreeing within 10%.

others (21,22). Presumably, the interaction of blood components like lipoproteins (23) with the liposomes is responsible for the observed enhanced release of the drug from its carrier. In a recent paper, Finkelstein and Weissmann (24) obtained quantitatively comparable results in measuring the leakage of radioactive inulin from multilamellar vesicles, consisting of PC, cholesterol (in different amounts) and dicetylphosphate, in the presence or absence of

serum. We do not know the rates at which Ara-C will be released from either type of liposome inside the peritoneal cavity. Upon injection, the liposomes are likely to undergo the influences of interaction with lymph. Lymph resembles plasma in composition but has a 30-50% lower protein content (25). It seems therefore reasonable to expect that drug-leakage from liposomes in lymph will not exceed leakage in plasma. There are several other uncertainties concerning the fate of liposomes in the peritoneal cavity: for instance, if and how liposomes interact with the mesothelial cells lining the cavity, is unknown. Peritoneal macrophages will presumably endocytose liposomes (26,27). We expect, however, that Ara-C introduced into macrophages by endocytosis of its carrier, would largely be degraded intracellularly. A tentative interpretation of the observed increase in antitumor-effect of Ara-C, resulting from its encapsulation within liposomes would then read as follows: the drug-laden liposomes reach in some way and to some extent the tumorous spleens and act, all along the way, as a depot from which the drug is released at a rate which depends on the vesicle-cholesterol content, and/or interact directly with the tumor cells to transfer the drug into the cells. Presumably, the liposomes are drained from the peritoneal cavity to the circulation via the lymphatics of the diaphragm and the large efferent lymphatics, thoracic duct and right lymphatic trunk. Once delivered into the circulation, the multilamellar liposomes will presumably be caught rapidly by the reticulo-endothelial system (28). It should be pointed out here (see also Materials and Methods) that, on the seventh day after inplantation of the tumor, hardly any reticulo-endothelial cells can be observed in the lymphosarcomatous spleen. As long as Ara-C remains inside liposomes, it is probably well protected against degradation and inactivation by deoxycytidine deaminase (10, and Refs. therein). Tissue- and plasma levels of this enzyme have been reported to be rather low in mice, as compared to humans (29). We do not know, however, whether this holds true for lymphosarcoma-bearing mice.

In a trial to - at least partially - check the probability of the course of events proposed above, we investigated whether liposomes would be able to deliver low molecular weight FITC-dextran (5) to the neoplastic spleen. The compound was entrapped in multilamellar liposomes, made up of PC, cholesterol, and PS (molar ratio 5 : 4 : 1 or 7 : 2 : 1) which carried [*Me*-^{14}C]PC as a vesicle membrane marker. Two hours after intraperitoneal injection of either free dextran plus either type of empty, radiolabeled vesicles, or of either

TABLE I

IN VIVO UPTAKE OF FREE OR LIPOSOME-ENTRAPPED FITC-DEXTRAN BY SPLEEN LYMPHOSARCOMA CELLS

Preparation	dose FDX (nmoles)	dose total lipid (μmoles)	LS cell-assoc. FDX* (pmoles/10^6 cells)	LS cell-assoc. vesicle lipid** (nmoles/10^6 cells)	M.R.***
Free FDX	210	-	0.21 ± 0.04 (0.013%)		-
Free FDX plus [^{14}C]MLV (5:4:1)	229	2.9	0.19 ± 0.02 (0.015%)	0.41 ± 0.10 (2.4%)	-
Free FDX plus [^{14}C]MLV (7:2:1)	229	3.4	0.19 ± 0.02 (0.014%)	0.68 ± 0.25 (3.0%)	-
FDX in [^{14}C]MLV (5:4:1)	229	2.9	6.6 ± 1.0 (0.52%)	0.43 ± 0.10 (2.7%)	0.0153 [0.079]
FDX in [^{14}C]MLV (7:2:1)	193	3.4	6.9 ± 2.7 (0.54%)	0.56 ± 0.30 (2.5%)	0.0125 [0.057]

* total cell associated FDX, measured in presence of 1% (v/v) Triton X-100

** based upon [^{14}C]PC uptake

*** molar ratio of cell-associated FDX to -total vesicle lipid; the corresponding molar ratios of the original vesicle preparations are given in square brackets

Abbreviations; MLV: multilamellar vesicles; FDX: FITC-dextran; LS: lymphosarcoma.

Legend to Table 1

Animals were injected intraperitoneally with 0.5 ml of 0.135 M NaCl/0.010 M HEPES (pH 7.4) containing either free or liposome-entrapped FITC-dextran. The liposomes consisted of PC, cholesterol and PS (molar ratio 5 : 4 : 1 or 7 : 2 : 1) and carried 0.1 µCi of [*Me*-^{14}C]PC (s.a. 2.2 Ci/mole) per µmole of total lipid. Two animals were injected with buffer only (for determination of fluorescence - plus scatter background). After 2 hours, the animals were sacrificed. The spleens were excised, rinsed in Hanks' basal salt solution, minced and passed through cheese cloth. The resulting cell suspensions were freed from erythrocytes by repeated washings (in 0.135 M NaCl/0.010 M HEPES, pH 7.4) and centrifugations (1000 x g; 60s). The washed cells were then taken up in 3 ml of 0.135 M NaCl/0.010 M HEPES buffer, and cell-associated FITC-dextran plus - [^{14}C]PC-radioactivity were determined as described in Materials and Methods. Cell counts were done with a Bürker hemocytometer, and cell viability was checked: > 90% (trypan blue exclusion). Values are given as averages of values obtained from two (in case of free FITC-dextran) or four (with liposome-entrapped dextran) animals, ± S.D. The average recoveries of the injected dose are listed in brackets, an refer to the total number of isolated cells, per spleen.

type of FITC-dextran-containing radiolabeled vesicles, spleens were taken out, lymphosarcoma cells were isolated, and the amounts of cell-associated dextran and cell-associated [^{14}C]PC-derived radioactivity were determined. The results are shown in Table 1. Dextran, administered entrapped in liposomes, is found cell-associated to a 30-fold higher extent than free dextran, whereas the amount of cell-associated [*Me*-^{14}C]PC-derived radioactivity seems roughly equal for all preparations used. The difference in cholesterol content between the vesicle-types used is not reflected in the amounts of FITC-dextran, which are correspondingly found cell-associated. With either type of vesicle the molar ratio of cell-associated markers (dextran/vesicle lipid, based upon [^{14}C]PC) is reduced 5-fold, as compared to the respective ratios in the original vesicle preparations. This may result from considerable leakage of the entrapped fluorophore from the liposomes during their stay in the body (see above) and/or from transfer/exchange of the radiolabeled lecithin between vesicles and - ultimately - spleen lymphosarcoma cells. Such transfer/exchange of radiolabeled lipid could be mediated through blood components, like high density lipoproteins (23) or occur upon association of liposomes with cells (*cf.* Chapter III). At present we do not know to what extent both processes, leakage and transfer/exchange of phospholipid, are involved in lowering the molar ratio of cell-associated markers. Fluorescence microscopy on the cells, isolated after *in vivo* "treatment" with dextran-containing liposomes, revealed that most of them displayed faint peripheral fluorescence, indicating the presence of FITC-dextran at the cell surface, either free or entrapped in liposomes. Most cells furthermore showed punctate fluorescence, against a faint, egally fluorescent background. The nuclei mainly appeared as relatively dark pits, indicating that the punctate fluorescence probably results from endocytosis of released dextran or dextran-containing vesicles. Lymphosarcoma cells from mice that had been injected with free dextran were barely visible under the fluorescence microscope. We are currently investigating the ultrastructural localization of free- or liposome-encapsulated horse radish peroxidase in the lymphosarcoma spleen.

In order to have an impression of the association of multilamellar liposomes with some other major organs, we intraperitoneally injected vesicles containing [^{125}I]-labeled PVP* (Table 2). For liver and spleen, both total uptake,

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* PVP: polyvinylpyrrolidone

TABLE 2

ORGAN DISTRIBUTION OF FREE AND LIPOSOME-ENTRAPPED [^{125}I]-LABELED POLYVINYLPYRROLIDONE UPON INTRAPERITONEAL INJECTION IN LYMPHOSARCOMA-BEARING MICE

Lymphosarcoma-bearing mice were intraperitoneally injected with 1.1 μCi [^{125}I]-labeled PVP (s.a. 46.3 μCi/mg), either mixed with, or entrapped in 6.25 μmoles of total liposomal lipid, in 0.5 ml 0.135 M NaCl/0.010 mM HEPES (pH 7.4). The multilamellar liposomes were composed of PC, cholesterol and PS (molar ratio 5 : 4 : 1). After 60 min, animals were killed by cervical dislocation and the organs were dissected out. Subsequently, the organs were rinsed in 0.25 M sucrose, blotted dry with filter paper and weighed. Organ-associated ^{125}I-gamma emission was determined as described in the Materials and Methods section. Values are given as averages, obtained from 4 animals, ± S.D. PVP: polyvinylpyrrolidone

Organ	liposome-entrapped [^{125}I]PVP			free [^{125}I]PVP		
	Organ wet weight(mg)	Organ uptake*	Organ spec. uptake**	Organ wet weight(mg)	Organ uptake*	Organ spec. uptake**
Spleen	797 ± 7	7.8 ± 0.6	9.8 ± 0.7	810 ± 78	2.3 ± 0.6	2.8 ± 0.5
Liver	1782 ± 87	21.4 ± 2.2	12.0 ± 1.5	1868 ± 201	4.7 ± 1.1	2.5 ± 0.5
Kidney	148 ± 14	0.7 ± 0.1	4.6 ± 0.7	142 ± 6	0.6 ± 0.2	4.5 ± 1.5
Heart	108 ± 4	0.3 ± 0.1	2.9 ± 0.4	97 ± 8	0.2 ± 0.1	1.7 ± 0.7
Lung	150 ± 36	0.8 ± 0.2	4.9 ± 0.3	177 ± 48	0.5 ± 0.2	3.0 ± 0.7
	recovery	31.0 ± 3.2		recovery	8.3 ± 2.2	

* % of administered dose

** % of administered dose per g wet organ weight

expressed as % of dose per organ, as well as specific uptake, expressed as % of dose per gram wet organ weight, are found to be significantly higher with the liposome-entrapped marker than with the free marker. About 29% of the dose of entrapped PVP is recovered from spleen plus liver, whereas for the free marker the corresponding recovery amounts to only approx. 7%. We emphasize that, in contrast to the values given in Table 1, the stated uptake values refer to the amounts of radioiodinated PVP which are recovered from whole organs including blood therein, and thus represent a "snap-shot" impression of the efficacy of liposomes to convey the marker from the peritoneal cavity to the circulation. Recently, Kooistra (30) demonstrated that the commercially available [^{125}I]PVP consists of a mixture of PVP-molecules with different molecular weights (range 8 000 - > 84000) and suggested , that the low molecular weight fraction is subject to renal excretion upon intravenous injection into rats. Since we used the commercial "crude" PVP, it is possible that a minor part of the difference in recovery (Table 2) between the liposome-entrapped and free marker must be ascribed to loss of low-molecular weight PVP, derived from the intraperitoneally injected free PVP, into the urine. Very recent experiments (31) from our laboratory indicate that in normal, healthy mice virtually all intraperitoneally injected PVP, either free or liposome-encapsulated, has been cleared from the peritoneal cavity within 4 hours after injection. Experiments regarding such clearance in lymphosarcoma-bearing mice are conducted at present.

DISCUSSION

The results from this pilot study offer circumstantial evidence that intraperitoneally injected, multilamellar liposomes can serve as vehicles for antineoplastic drugs. This conclusion is mainly based upon the finding that, following intraperitoneal administration, liposome-entrapped Ara-C ultimately outranks an equal dose of free Ara-C in causing an inhibition of the DNA-synthesis in mouse lymphosarcomatous spleen. The liposome-mediated increase in apparent efficacy of the drug is most clearly expressed by the extension of the duration of the inhibition, and seems to be inversely related to the liposomal cholesterol content. The increase in drug efficacy may result from sustained release of the drug from liposomes at a rate which depends (inter alia) on the liposomal cholesterol-content and/or from direct physical interaction between liposomes and "target" cells. Apparently, during the first 100 min after injection DNA-synthesis is equally inhibited by Ara-C given as free drug

and Ara-C administered encapsulated in liposomes. At present we cannot explain this phenomenon. Presumably, upon either way of administration the amount of undegraded Ara-C that ultimately enters into the tumor cells is roughly the same during this period. At the dose* given, this could be a mere coincidence, resulting from a similar net outcome of the influences of a number of factors which determine the pharmacokinetics of Ara-C on one hand and the kinetics of the drug-containing liposomes on the other. For example, we do not know how much non-encapsulated Ara-C could reach the diseased spleen by direct passage through the linings of the peritoneal cavity. If the laws of diffusion would apply to such passage, one would expect that, in case Ara-C is given as a free drug, the absolute amount of drug reaching the lymphosarcoma cells in the spleen would be higher than in case an equal dose of liposome-encapsulated Ara-C is administered. In the latter case only a fraction of the drug, *i.e.* those molecules that have leaked out of the carrier, would be available to diffuse out of the peritoneal cavity. In this context it is of interest, that Ara-C has been reported to accumulate in the spleen, when given intraperitoneally to mice (32). Furthermore, we do not know at present whether and to what extent the drug might be degraded by deaminase-activity even before entering the bloodstream, and if so, at which drug concentrations the enzyme would display a maximal substrate-turnover rate. Possibly, simultaneous intraperitoneal administration of either free or encapsulated Ara-C and tetrahydrouridine, a potent inhibitor of the deaminase (32), might lead to a different picture during the first 100 min after injection. In any case, we would expect to observe some difference between free and encapsulated Ara-C with respect to its potential to inhibit DNA-synthesis in the tumor during this period, if the drug-dose is varied.

The rate at which lymph is drained from the peritoneal cavity (*cf.* 33) is likely to codetermine the kinetics of an intraperitoneally deposited drug, either free or entrapped in liposomes. The experiments depicted in Tables 1 and 2 provide circumstantial evidence that liposomes do not constitute a merely intraperitoneally localized depot. In contrast to Ara-C, both FITC-dextran and [^{125}I]-labeled PVP are metabolically inert (34,30); their ultimate tissue distribution is determined by physical factors. FITC-dextran and

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* In case of free Ara-C, the dose used (13.7 mg/kg body weight) does not cause maximal inhibition of DNA-synthesis in the tumor (*cf.* Ref. 3).

[^{125}I]-labeled PVP that leak out of the liposomes during their stay in the peritoneal cavity (and beyond) are expected to follow the same route as taken by the corresponding non-encapsulated compounds. The finding (Tables 1 and 2) that the encapsulated markers end up in spleen and liver to a much greater extent than the non-encapsulated ones therefore strongly suggests that the liposomes either pass through the peritoneal mesothelium and/or are carried to the blood circulation with the lymph, more or less representing an - internal - intravenous infusion system. Such considerations can of course be readily extrapolated to liposomes which contain Ara-C. The fluorescence microscopic image of lymphosarcoma cells following the intraperitoneal "treatment" with FITC-dextran-containing liposomes furthermore indicates that a direct - *i.e.* through liposome-cell contact- delivery of the drug into the cells cannot be excluded. Experiments in which isolated lymphosarcoma cells were incubated *in vitro* with *small unilamellar* vesicles, showed that carboxyfluorescein (*cf.* 5) can be transferred directly from vesicles into the cells. At the same time, however, considerable amounts of dye became cell-associated in a highly quenched state, indicating endocytosis or adsorption of vesicles. (A.J.B.M. van Renswoude and A.W.T. Konings, unpublished data).

It has been suggested that intraperitoneally injected liposomes can be recovered intact from the bloodstream (35). However, no thorough studies on the route taken by liposomes under such circumstances nor on the rate at which the vesicles would enter the bloodstream, have been published. As a first approach to try and quantitate a possible transport of vesicles from the peritoneal cavity to the blood circulation, canulation of the large efferent lymphatics with proper assay of thus collected lymph for the presence of previously intraperitoneally injected liposomes, could be considered. Alternatively, blockade of the peritoneal lymph drainage by ligation of the large efferent lymph vessels should give some impression as to the extent at which liposomes or free markers might leave the peritoneal cavity by passage through the peritoneal membrane. It should be stressed that until the present time no convincing evidence concerning a passage of intact liposomes through anatomical barriers - such as capillary endothelium and peritoneal mesothelium - has been produced.

During the course of this work, some papers dealing with the effects of encapsulation of Ara-C within liposomes on its antitumor efficacy, were published (36-39). The results reported therein agree well with our findings.

In addition, it was shown by Rustum *et al.* (36) that multilamellar vesicles were more effective in enhancing the effect of Ara-C against L1210 cells, than unilamellar vesicles, irrespective of vesicle-charge, and that liposomes per se were virtually non-toxic: upon intravenous injection the dose of lipid lethal to 50% of the mice was found to exceed 5 g/kg body weight. Using essentially the same experimental tumor model system, Kobayashi *et al.* (37) reported that out of three lipid compositions tested, multilamellar vesicles consisting of sphingomyelin, stearylamine, and cholesterol (molar ratio 20 : 2 : 15) were found to enhance the effect of Ara-C most effectively. This was attributed to the very low *in vitro* leakage rate of the drug from this kind of liposomes. Investigations in our laboratory (40,41) as well as from others (24) indicate that liposomes consisting of sphingomyelin and relatively high amounts of cholesterol are also highly stable in the presence of plasma, most likely mainly because of very tight packing of the constituent lipids and/or a decreased susceptibility towards attack by plasma components such as high density lipoprotein (23). Our results with liposomes consisting of PC, PS and different amounts of cholesterol indicate that the "tighter" liposome ultimately does not necessarily have to be the more effective one. Much will depend on the basic pharmacokinetics of the drug chosen, as well as on the localization of the tumor.

In some of the liposome - Ara-C studies quoted above, as well as in our experiments, multilamellar liposomes were used mainly because of their higher trapping efficiency for water-soluble compounds, as compared to small unilamellar vesicles (*cf.* 36). A much higher trapping efficiency (up to 4 µl of aqueous volume per µmole of total lipid) can be obtained with reverse phase evaporation vesicles (42). The use of such vesicles in efforts to enhance the effectivity of antitumor drugs is currently under study in our laboratory.

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C H A P T E R VI

THIN-LAYER CHROMATOGRAPHY WITH AGAROSE GELS: A QUICK, SIMPLE METHOD FOR EVALUATING LIPOSOME SIZE

SUMMARY

Thin-layer gels can be made with agarose and used to assess within a few minutes the efficiency with which multilamellar vesicles are converted to small unilamellar ones by sonication. A fluorescent lipid marker or vesicle-encapsulated solute permits continuous monitoring of the chromatography.

Advantages over agarose gel column chromatography include speed of analysis, small sample size, the possibility of running multiple samples simultaneously, and direct accessibility to fluorescence microscopy. This approach should also be useful in the study of liposome-lipoprotein interactions and in affinity chromatography of liposomes.

Small unilamellar vesicles are used in a variety of physical-chemical, cell biological, and medical studies (1-3). They are usually obtained by sonication of multilamellar lipid dispersions, and it is generally important to know how effective the sonication was in producing small unilamellar vesicles. The answer has most often been obtained by agarose gel chromatography in a column, according to methods developed by Huang (4). However, column chromatography has several drawbacks for analytical use: first, the reduction in vesicle size is assessed only after all of the lipid has been eluted from the column; second, only one sample at a time can be analyzed per column; third, relatively large sample size is required (an advantage for preparative purposes). In this report we describe the development of thin-layer chromatographic methods for quickly assessing the efficiency of liposome* sonication. Other, broader areas of application for this technique will become apparent as well.

Thin-layer chromatography with sephadex gel was first described by Determann for the determination of protein molecular weight (5). For our purpose

* In this report the terms "liposome" and "vesicle" will be used interchangeably.

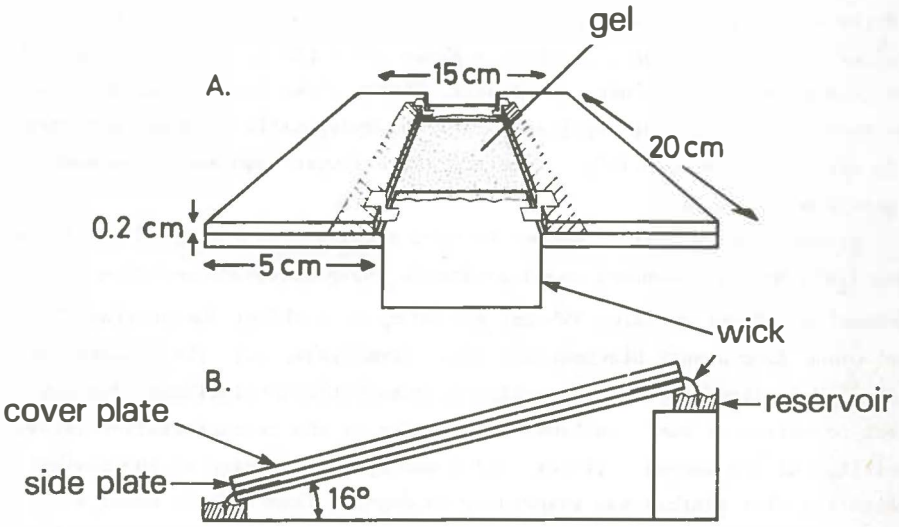

Fig. 1. *The gel bed support.* A. End-view. The side plates are attached to the base plate by strips of Scotch magic tape (striped areas) folded back on themselves with edges away from the gel; absolute water-thightness of the tape-seal is necessary to prevent leakage of eluent and distortion of the flow pattern. This can be guaranteed by applying silicone grease between side- and base plate behind the tape. B. Position of the support during chromatography. For the glass plates we used sections of pre-scored 20 x 20 cm chromatography plates (Analtech, Newark, DE), with the silica gel removed. The 16" angle is not critical; a larger angle would still allow a good (and faster) separation.

Sepharose 4B (Pharmacia), an agarose gel with the appropriate range of size discrimination, was used instead. Fig. 1A shows the construction of a support for the gel bed, and Fig. 1B shows the position of the support during a chromatographic run. The support was made from thoroughly cleaned glass plates, held together by tape. Sheets of dry Whatman No. 1 filter paper (4.6 x 10 cm) were attached to the bed support with masking tape. With the support horizontal, approximately 3 ml of a slurry of Sepharose 4B (33 mg dry particle weight per ml of the desired elution buffer) was spread onto the bed support using a spoon, starting from the center of the bed and moving toward the filter paper wicks. Additional slurry was used to assure contact between the wicks and gel bed. Uptake of excess buffer by the wicks took 5 - 10 minutes. Any disturbance of the gel during this stage resulted in a layer of uneven thickness, but such

uneveness had only minor effects on the elution pattern. As soon as the bed had dried to a dull, matted appearance, it could be readied for use by cutting the wicks to 6 cm in length, mounting a glass cover plate, and immersing both wicks in reservoirs containing the eluent. Eluent flows from the upper to the lower reservoir because of capillarity and the hydrostatic pressure difference. No special precautions (*e.g.*, a moist chamber) were required to prevent the gel from drying out.

For preparation of test liposomes we used a mixture of 2.5 mg of the fluorescent lipid N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (excitation 470 nm; emission 545 nm) and 50 mg of dioleoyl phosphatidylcholine (both from Avanti Biochemicals Inc., Birmingham, AL). The presence of 5 mole % N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine did not affect formation of small unilamellar vesicles or the characteristics (size, stability) of the formed vesicles. Lipid purity was assessed as in previous studies (6). The mixture was evaporated to dryness from benzene under a stream of argon and lyophilized overnight. The lipid film was dispersed by vortex-mixing in 5 ml of phosphate-buffered saline (pH 7.4). Part of the resulting multilamellar vesicle preparation was sonicated under argon at about 30 ^{o}C for periods of up to one hour with a Heat Systems sonicator equipped with a titanium microprobe.

To test the resolution of the Sepharose 4B thin-layer, we mixed vesicles sonicated for 1 hr with unsonicated ones, added purified (6) carboxyfluorescein (Eastman Kodak Co., Rochester, NY) to a final concentration of 20 μM, and chromatographed the resulting mixture. The separate constituents were run simultaneously on the same plate. After 75 minutes the chromatograph was photographed under long-wavelength ultraviolet light (see Fig. 2A). There was excellent separation of multilamellar from small unilamellar vesicles while separation of the latter from carboxyfluorescein was relatively poor. Some of the unsonicated vesicles remained at the origin, in part representing vesicles too large to enter the gel.

Fig. 2B shows the spots obtained with liposome preparations subjected to increasing times of sonication. Samples sonicated for 2, 5, 15 and 60 minutes, had, respectively, a turbid, heavily opalescent, faintly opalescent and clear appearance. Obviously, a 15-minute sonication was not sufficient to convert all multilamellar vesicles into small unilamellar ones. In contrast to Fig.

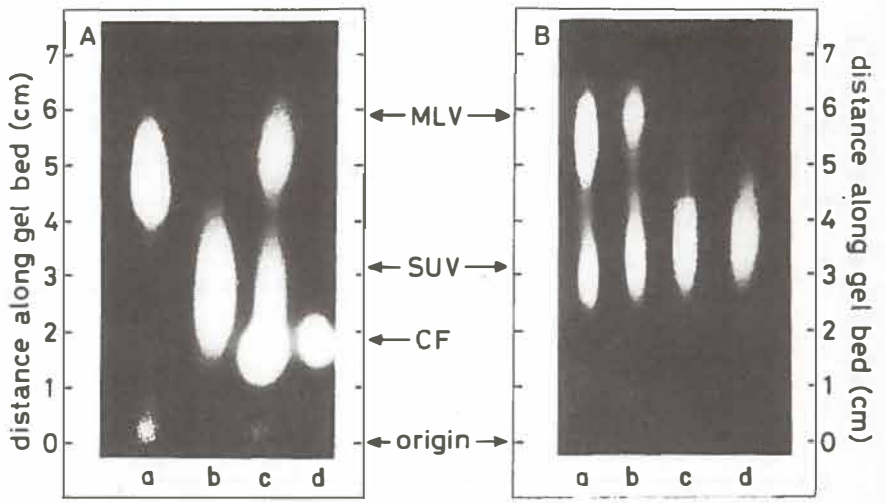

Fig. 2. *Thin-layer gel chromatograms of liposome preparations on Sepharose 4B.* Liposomes were prepared as described in the text. A. Resolution of multilamellar vesicles (MLV) and free solute. 10-µl samples (100 µgr total lipid) of multilamellar (a), small unilamellar vesicles (b), 1 : 1 (w/w) mixture of multilamellar and small unilamellar vesicles plus 20 µM free carboxyfluorescein (c), and 20 µM free carboxyfluorescein (d) were applied using a 5-µl Oxford micropipette. Careful application of the spot was important to a good separation, and the Oxford pipette was considerably easier to control than others tried. Chromatography was stopped after 69 minutes by levelling the plate and removing the paper wicks. B. Distribution of liposome size after different times of sonication. 10-µl samples of liposome preparation sonicated for 2 min (a), 5 min (b), 15 min (c), and 60 min (d) were applied to the gel. Chromatography was stopped after 73 min. The sharp central streaks are due to scattering from scorings on the glass.

1A no lipid remained at the origin, indicating that sonication for as little as 2 minutes sufficed to break up very large structures (and to form substantial amounts of small unilamellar vesicles).

For quantitative analysis a mixed vesicle preparation similar to that in Fig. 2A was chromatographed. The thin-layer was then scanned for fluorescence. As shown in Fig. 3, multilamellar vesicles were separated from small unilamellar ones within a total distance of about 6 cm.

The fluorescent spots on a similar gel were studied *in situ* with a fluorescence microscope (Fig. 4). At the origin (A) some very large liposomes

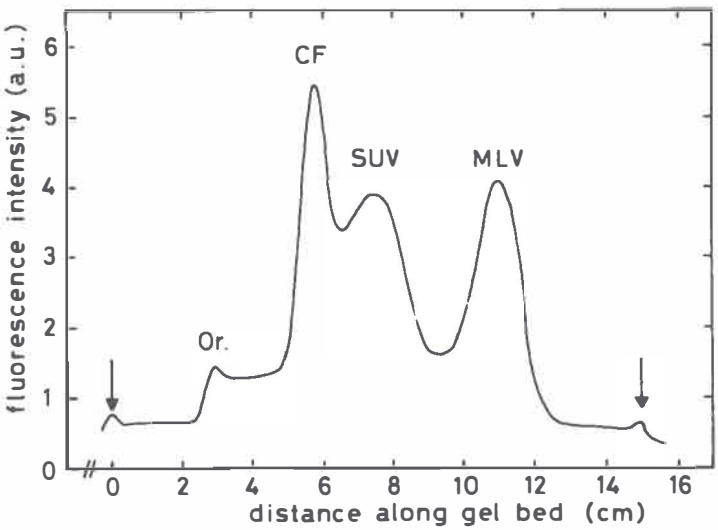

Fig. 3. *Fluorescence scan of a thin-layer Sepharose 4B gel chromatogram.* The scan was obtained with a fiber-optic thin-layer scanner attached to a fluorimeter (both from American Instrument Co., Silver Spring, MD). Scan-speed was 1.5 cm/min with excitation at 470 nm and emission at 520 nm. Ten µl (100 µgr total lipid) of a 1 : 1 (w/w) MLV/SUV (multilamellar/small unilamellar vesicle) preparation (see text), containing 4 µM free carboxyfluorescein was applied to the gel. Chromatography was stopped after 93 min. Arrows indicate ends of the gel bed. Or.: Origin, a.u.: arbitrary units.

(arrow) remained, and all of the gel beads had fluorescent rims, probably because of adsorption of vesicle lipid. In the spots containing carboxyfluorescein (B) and small unilamellar vesicles (C), the gel beads appeared uniformly fluorescent, indicating free dye and vesicles, respectively, inside the beads. At the outer edge of the multilamellar vesicle spot (D), by contrast, fluorescence was mainly observed outside the beads, as expected. Some gel beads had brightly fluorescent rims, probably corresponding to adsorbed vesicle lipid. Vesicles also appeared between beads (arrow).

We also chromatographed vesicles with ^{14}C-labelled lipid, but the results were less satisfactory; if the gel was dried for scanning of the tracer, the elution pattern became somewhat blurred, and the gel surface tended to crack. In any case, fluorescent labeling has the advantage over radiotracers that the chromatographic process can be monitored continuously. As an

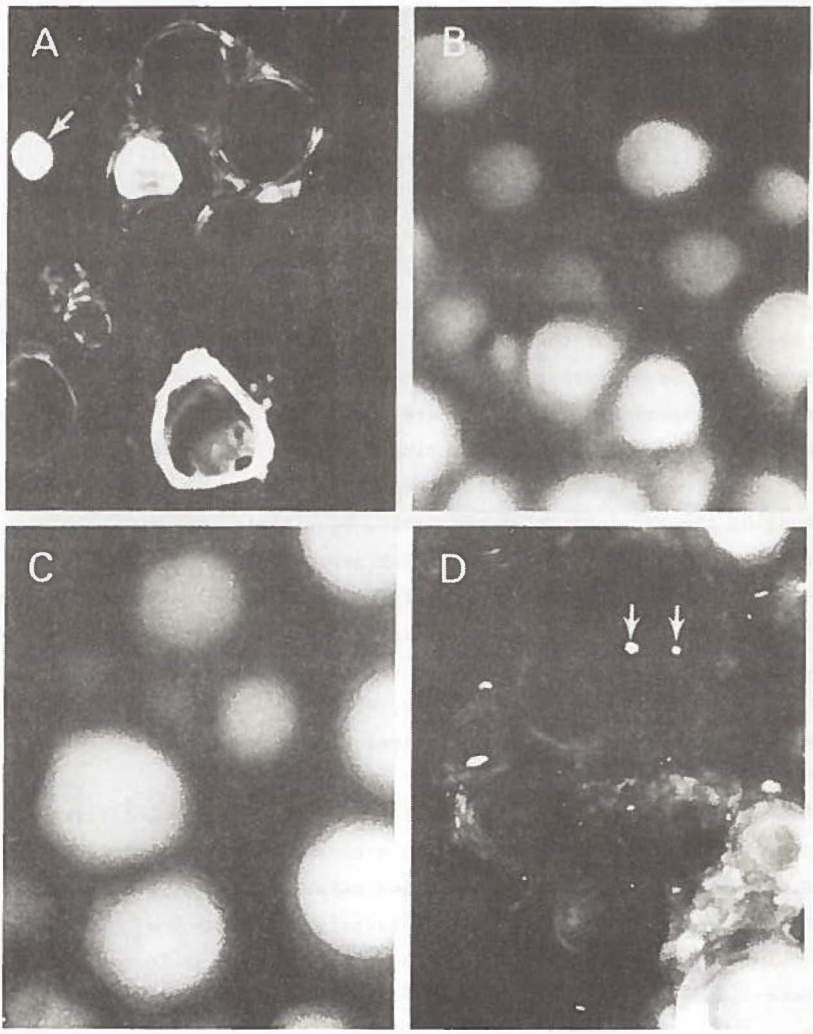

Fig. 4. *Fluorescence microscopy of a Sepharose 4B thin-layer gel after chromatography of the mixture used for the scan in Fig. 3.* Pictures were taken through a Zeiss Universal fluorescence microscope with filter combination for fluorescein. A: origin; B: carboxyfluorescein spot; C: small unilamellar vesicle spot; D: outer edge of multilamellar vesicle spot. Arrows: see text. Mag. 100x.

alternative to intrinsic fluorescent dyes (*e.g.*, N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine in our experiments) vesicles could be labeled with lipophilic fluorophore just prior to chromatography.

Advantages of the thin-layer method over conventional column gel chromatography include 1) the possibility of running several samples simultaneously on the same gel; 2) speed. A gross impression of the vesicle size distribution can be obtained after as little as 10 minutes of elution time; and 3) the very small size of the samples applied. In the experiments reported here we applied 100 µgr of vesicle lipid, which gave very bright fluorescent spots (Fig. 2); easily detectible on fluorescence scans (Fig. 3). The data indicate that the sensitivity of the method is 1 - 10 µgr of total lipid, depending on the conditions of the experiment and the fluorescent probe used.

After the experiments reported here, we found that the speed of chromatography could be doubled by substituting a cross-linked agarose (Sepharose CL-4B) for Sepharose 4B. Solvent flow through the bed could also be manipulated by changing the inclination of the plate, by altering reservoir levels, or by changing the thickness of the wicks. The separation of small unilamellar vesicles from low molecular weight solutes like carboxyfluorescein was improved considerably by combining a dextran stacking gel (*e.g.*, Sephadex G-50) with a Sepharose 4B separation gel. This was done by simultaneously pouring the two gels onto the support, separating them initially with a thin polystyrene blade positioned perpendicular to the gel. After 3 minutes the blade was carefully withdrawn.

With inclusion of sodium azide (0.1 %) and adequate pH buffering in the eluent, the thin-layer can be run like a conveyor belt for at least several weeks of routine use; previously applied material simply runs into the lower reservoir. In addition to its use in assessing efficiency of sonication, agarose thin-layer gel chromatography should prove immediately applicable to liposome-lipoprotein interactions (7) and to affinity chromatography of liposomes.

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A.J.B.M. van Renswoude, R. Blumenthal and J.N. Weinstein, Biochim. Biophys. Acta, in press.

C H A P T E R VII

CONCLUDING REMARKS

The results of the experiments described in Chapters II-IV allow to construct a hypothesis on the mechanisms of *in vitro* interaction between Zajdela ascites hepatoma cells and small unilamellar vesicles*, consisting of PC, cholesterol and PS (molar ratio 5 : 4 : 1). Presumably, the initial interaction between vesicles and cells involves the binding of vesicles to two - at least partly - kinetically distinguishable classes of sites at the cell surface: transfer- and adsorption sites. Binding to a transfer site results in direct entry of the vesicle-contents into the cytoplasm, probably via a membrane fusion-like mechanism (see below). We have produced circumstantial evidence that binding of vesicles to transfer sites must be a reversible process. However, vesicles or the remnants thereof seem to remain cell-associated after transfer of their contents into the cytoplasm has been accomplished. We therefore propose that the transfer sites are cleared from vesicles in a lateral fashion, and at a rate which is as yet unknown. "Cleared" vesicles could then be considered to end up bound to cell surface areas which serve as a "dumping" region.

Binding of a vesicle to an adsorption site might result in rapid leakage of the vesicle-contents into the surrounding medium. Such leakage occurs with vesicles that possess a relatively high cholesterol content and is presumably initiated through the interaction of cell-surface glycoproteins with the vesicles (see Chapter IV). A schematic view of the interaction hypothesis is given in Fig. 1. For reasons of simplicity the "dumping" region is suggested to be structurally and functionally identical to the adsorption site. It should be emphasized that the model displayed above represents merely a working-hypothesis; its verification must await further experimentation. The structural and functional properties as well as the localization of the different sites at the cell surface are unknown at present. Blumenthal *et al.* (1) studied the *in vitro* interaction of small unilamellar dioleoyllecithin vesicles with human peripheral lymphocytes, and reported the discovery of two

* the terms "vesicle" and "liposome" are used as synonyms.

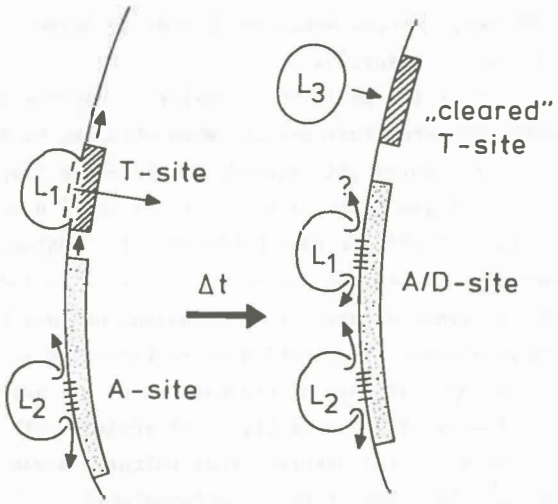

Fig. 1. *Schematic view of a hypothetical two-site vesicle-cell interaction model.* For description see text. T-site: transfer site (hatched area); A-site: adsorption site (dotted area); A/D-site: combined adsorption/"dump" site. The curved arrows indicate possible leakage of vesicle contents.

different classes of sites through which vesicle-carboxyfluorescein is transferred into the cells: saturable and non-saturable transfer sites. They suggested that the saturable sites might be identical to bare lipid patches at the cell surface. Recently, Hoekstra *et al.* (2) proposed that the surface of the rat hepatocyte contains at least two different classes of sites for interaction with small unilamellar vesicles, consisting of PC, cholesterol and dicetylphosphate (molar ratio 2 : 1.5 : 0.22). Sites for transfer of vesicle-carboxyfluorescein were found to behave kinetically different from stable adsorption sites; fetal calf serum was shown to considerably decrease the amount of vesicles bound to adsorption sites, whereas dye-transfer was not affected by the presence of serum. Disregarding the difference in lipid composition between the vesicles used in Chapters II and III and those used by Hoekstra *et al.*, it is noteworthy that, as compared to Zajdela cells, hepatocytes take up considerably larger amounts of vesicle-carboxyfluorescein, both via transfer and via stable adsorption to the cell-surface. Stable adsorption of vesicles to cells has been reported by a number of investigators (3-10).

Although the experimental conditions as well as the types of vesicles and cells used differ widely among these studies, the general picture is that "solid" vesicles and positively charged vesicles display relatively high degrees of stable adsorption to cell-surfaces.

An important question is by which mechanism transfer of vesicle-contents occurs. We have been using the term "fusion-like" when alluding to our view of the possible nature of this mechanism. As already mentioned in Chapter I, fusion of a vesicle with the plasma membrane is likely to involve molecular intermixing of the constituent lipids of both bilayers, plus coalescence of the inner aqueous compartments of vesicle and cell. We have also indicated that, in order to be able to speak of vesicle-cell fusion, one has to demonstrate the primary intracytoplasmic localization or -activity of a formerly vesicle-entrapped substance (*cf.* Ref. 4). At present, there are several important lines of evidence in favour of the possibility of vesicle-cell fusion. For example, Ostro and co-workers (11) observed that cultured human epithelial carcinoma cells, treated with liposomes carrying encapsulated rabbit globin messenger-RNA (mRNA), were stimulated to produce a globin-like protein. Similarly, Dimitriadis (12) demonstrated in an *in vitro* study that rabbit globin mRNA can be delivered by liposomes into the cytoplasm of mouse spleen lymphocytes and subsequently translated to produce globin-like proteins. Dimitriadis and Butters (13) showed that ricin-resistant BHK-cells became sensitive to the toxin if it was offered encapsulated in liposomes. Recently, Wilson *et al.* (14) succeeded in introducing infectious polivirus RNA into HeLa-cells and Chinese hamster ovary cells. Clearly, in all studies mentioned above, some form of vesicle-cell fusion must have occurred. The question is, however, whether such fusion is "complete", *i.e.* resulting in a complete merging of the vesicle into the plasma membrane (*cf.* Chapter I, Fig. 2). Wilson *et al.* (14) mention the possibility of passage of the poliovirus RNA through a highly permeable liposome-cell contact region. Such a region might consist of the closely apposed bilayers of vesicle and cell, and derive its enhanced permeability from a local perturbation of the molecular organization within the contacting membranes. It is tempting to speculate that a perturbation might result from the formation of non-bilayer structures like inverted micelles (15,16) within the apposed bilayers. Secondary conditions would then determine whether or not such a situation will lead to complete fusion. It can be calculated from our experiments (Chapter II) that the initial rate of transfer

of vesicle-FITC-dextran into cells is at least twofold slower than that of vesicle-carboxyfluorescein. This difference in initial transfer rate argues against "complete" vesicle-cell fusion as the mechanism through which vesicle-contents are delivered into the Zajdela cell. For this and other reasons (see below) we prefer to adopt - for the present time - the formation of an appositional membrane-complex as the underlying mechanism of transfer.

One way to get some impression of the degree of molecular intermixing of "fusing" vesicle- and plasma membranes is to investigate the lateral diffusability of fluorescent lipid analogues in the plane of the plasma membrane, using the fluorescence recovery after photobleaching (FRAP) technique. Fluorescent lipid analogues like 3,3'-dioctadecylindocarbocyanine (diI) and N-4-nitrobenzo-2-oxa-1,3-diazole-phosphatidylethanolamine (NBD-PE) can be incorporated in trace amounts into the vesicle-bilayer, and their vesicle-mediated association with the cell-surface can be visualized (18). Upon incubation of diI- or NBD-PE-containing small unilamellar vesicles, consisting of PC/PS (9 : 1) or of dipalmitoyllecithin, with various cell types, Szoka *et al.* (18) observed no lateral diffusion of either probe at the cell surface, although with some cell-types transfer of vesicle-carboxyfluorescein was seen. Similarly, upon *in vitro* incubation of P388 cells (a murine lymphoid cell line) with small unilamellar dioleoyllecithin vesicles, Blumenthal and colleagues (19) observed punctate, vesicle diI-derived fluorescence at the cell periphery. There was no fluorescence recovery after photobleaching of the probe at the cell surface, although the vesicles studied were capable of transferring carboxyfluorescein effectively into the cells. DiI, administered to the cells as an ethanolic solution, was found to diffuse freely in the plane of the plasma membrane. In preliminary experiments we recently observed punctate ring fluorescence on Zajdela cells following incubation with small unilamellar vesicles composed of PC/NBD-PE/cholesterol/PS (molar ratio 4.75 : 0.25 : 4 : 1). The observations mentioned above also suggest that "complete" fusion between vesicles and cells may not be necessary for the introduction of vesicle-contents into cells along a non-endocytotic route.

With regard to the appositional-membrane-complex hypothesis, it would be of interest to have an impression of the sizes of molecules that may be transferred from vesicles of a given lipid composition into the cytoplasm of a certain cell-type. This could be accomplished by studying the vesicle-mediated transfer of highly fluorescently labeled polymers as a function of their

molecular weight. Because of their metabolic inertness and their occurrence in various, defined molecular weight classes, FITC-dextrans could be considered suitable candidates for such purposes. The commercially available FITC-dextrans, like the species used in the experiments described in Chapter II, are fluorescently tagged to a degree too low to attain a level of self-quenching inside vesicles which is high enough to monitor low rates of vesicle-mediated transfer. Preliminary experiments indicate that coupling of dichlorotriazinyl-aminofluorescein (DTAF) (20) to N-(2-aminoethyl)carbamyl-methyl-(AECM) substituted dextrans (21) of various molecular weight produces highly fluorescent compounds, which are currently under investigation with regard to their self-quenching properties inside vesicles. If such DTAF*-AECM-dextrans will also prove to be metabolically stable, they might be very useful in tracing the fate of liposomes *in vivo* (*cf.* Chapter V).

During the studies described in this thesis, we have been trying to obtain evidence for the ability of liposomes to deliver a functionally active substance into the cytoplasm of Zajdela cells by incubating Zajdela cells and - in a later stage - isolated mouse spleen lymphosarcoma cells (see Chapter V) with liposomes containing ribonuclease. It was originally suggested to us by A.M. Kroon, that the enzyme, once delivered into the cytoplasm, might cause a rapid breakdown of messenger-RNA (mRNA). The resulting deterioration of the translation process might then be observed as a decrease in the incorporation of radiolabeled amino acids into *de novo* synthesized protein or in a collapse of the polyribosome profile. Bovine pancreas RNAse A was entrapped in small unilamellar vesicles, composed of PC/PS (molar ratio 9 : 1) and incubated *in vitro* with Zajdela cells for maximally 4 hours. The results were virtually negative: only 15% inhibition** of [^{14}C]leucine incorporation into *de novo* synthesized protein was observed. The polyribosome profile was found deteriorated but we could not exclude that this was caused by RNase, released from vesicles which were adsorbed to the cell-surface, during or after the cell-homogenization procedure. We realized that, although the enzyme was found to become cell-associated in an active form, the lack of effect on cytoplasmic protein synthesis could be due to (a combination of) several factors.

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* Apart from DTAF, a variety of other fluorescent- or radioactive marker groups might be readily coupled to the primary amino-groups of AECM-dextran.

** as compared to appropriate controls, such as: cells alone and cells plus empty vesicles premixed with free RNase.

Possibly, the enzyme:

a. -is not or insufficiently internalized into the cytoplasm.

b. -is internalized but inactivated by naturally occurring cytoplasmic RNase inhibitor (22).

c. -is internalized and remains active in sufficient amounts, but cannot reach the substrate, mRNA (distance? free-*vs.* membrane-bound polysomes?).

d. -attacks substrate, but only to a degree which does not lead to a noticeable overall inhibition of *de novo* protein synthesis (synthesis of small peptides at mRNA-fragments?).

e. -attacks substrate, but substrate is rapidly resynthesized.

Some of these possibilities could be practically excluded. For example, vesicle-cell incubation in the presence of Actinomycin-D, which inhibits RNA-synthesis (23) did not result in higher inhibition values than the maximal 15% stated above. This finding ruled out possibility e. No significant inhibition of leucine-incorporation was observed if the vesicle-RNase A was replaced by RNase T_1. In contrast to RNase A, RNase T_1 was found not to be inhibited by cytosol from Zajdela cells. Hence, possibility b seems less likely to be responsible for the lack of effect of RNase A. Polyacrylamide gel electrophoresis of postmitochondrial supernatants of Zajdela cells following incubation with RNase-containing vesicles in the presence of [^{14}C]-leucine, revealed that the molecular weight- and radioactivity-distribution of the isolated proteins was grossly the same as compared to those of control cells. This practically ruled out possibility d. The consideration that the translation process itself (*i.e.* the movement of ribosomes along the mRNA) could prevent the enzyme from attacking its substrate led us to pretreat cells with puromycin in order to cause a massive run-off of ribosomes. Upon subsequent incubation of such cells in puromycin-free medium, [^{14}C]leucine-incorporation rapidly (within 15 min) recovered to the values obtained with non-puromycin-treated cells, regardless of the presence (or absence) of RNase-containing vesicles during the incubation. Furthermore, no increase in the maximal value of 15% inhibition of leucine-incorporation was observed upon using multilamellar vesicles (see Chapter V) or reverse phase evaporation vesicles (24) instead* of small unilamellar vesicles for the encapsulation of

* multilamellar vesicles and reverse phase evaporation vesicles were found to entrap the enzyme about 3- and 10-fold more effectively than small unilamellar vesicles, based on entrapped volume per µmole lipid.

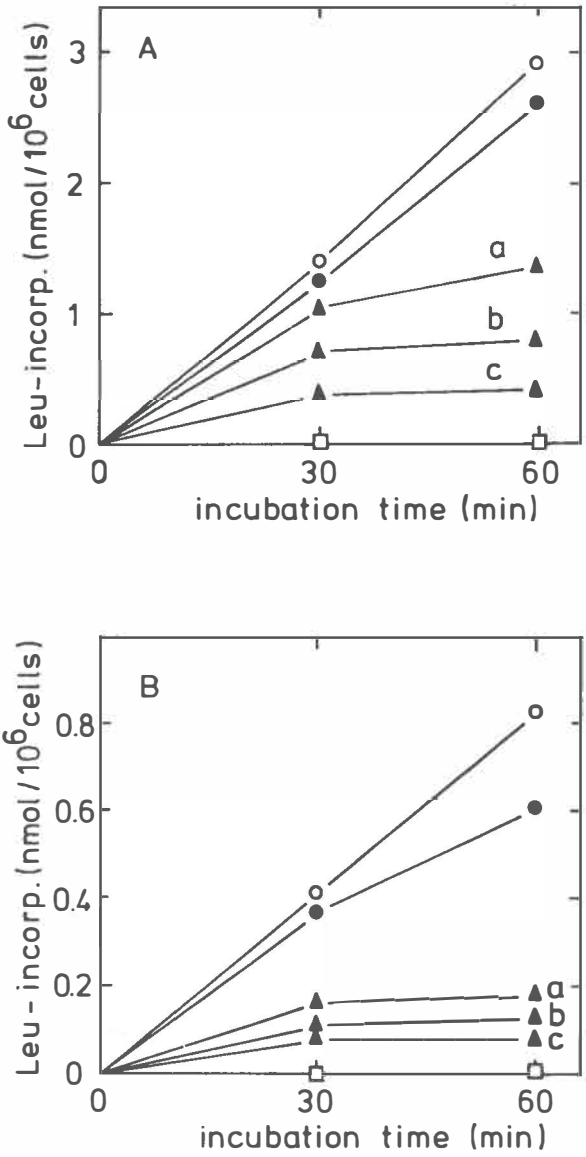

Fig. 2. *Inhibition of de novo protein synthesis in Zajdela cells (A) and lymphosarcoma cells (B) by liposome-entrapped ribonuclease.*

For legend to this Figure see next page.

Fig. 2. *Inhibition of de novo protein synthesis in Zajdela cells (A) and lymphosarcoma cells (B) by liposome-entrapped ribonuclease.* For isolation of Zajdela cells see Chapter II; lymphosarcoma cells were isolated as described in Chapter V, except that the cells were freed of contaminating erythrocytes by centrifugation (400 x g; 45 min) through a Ficoll-Paque^{R} (Pharmacia) cushion (27) Prior to incubation with vesicles cells were kept in serum-free RPMI-1640 medium for at least 30 min. Small unilamellar vesicles, consisting of bovine brain PS (Sigma) were generated essentially as described in Chapter II for PC/cholesterol/PS vesicles, in 0.135 M NaCl/ 0.010 M HEPES/0.001 M EDTA, pH 7.4 ("standard buffer"). For preparation of RNase-containing vesicles, PS was dispersed in a suitable dilution of a 100 mg/ml solution of RNase A (Sigma) in standard buffer. The sonication process (1 hr) did not significantly affect the activity of the enzyme (28). Vesicles were separated from non-entrapped enzyme by chromatography on Sephadex G-200, using standard buffer as an eluant. For either cell-type, 5 x 10^6 cells per ml were incubated (37 ^{o}C, shaking water bath, open polystyrene tubes) with 0.4 mM of vesicle lipid, in a final volume of 5.7 ml. Control incubations of cells alone, cells with 0.4 mM empty vesicles, 0.4 mM empty vesicles plus free RNase (16 µg/ml), and cells with 6 x 10^{-6} M anisomycin plus puromycin (100 µg/ml) were run simultaneously. The incubation mixtures consisted of a 3 : 1 (v/v) mixture of RPMI 1640 tissue culture medium and standard buffer, contained Ca^{2+} to a final concentration of approx. 4.3 mM, and L-[1-^{14}C]leucine (Radiochemical Centre, Amersham, U.K.; spec. act. 62 µCi/µmol) to a final specific activity of 1.8 µCi/µmol. The final concentration of total leucin in the incubation mixtures was 0.24 mM. After 30 and 60 min of incubation time, duplicate 1-ml samples were withdrawn from the incubations and pipetted into tubes containing 0.4 ml of a 1 : 2.5 : 0.5 (v/v) mixture of cold leucine (20 mg/ml), sodium deoxycholate (5%, w/v) and bovine serum albumin (50 mg/ml). 1.4 ml of 10% trichloroacetic acid was added immediately. The resulting precipitates were processed as described by Gijzel (29). The pellets were finally dissolved in 1 ml Lumasolve^{R}. 6 ml of Lipoluma^{R} was added and radioactivity was determined by liquid scintillation counting with a Nuclear Chicago MkII counter. Radioactivity values obtained from incubations of cells in the presence of anisomycin plus puromycin (less than 0.5% of the values obtained from cells incubated with empty vesicles) were substracted from all other radioactivity values. 250 pmol of incorporated leucine corresponds with 1 000 dpm. Each point represents the average of the values of a duplicate measurement agreeing within 10%.
The values for cells incubated without vesicles coincided with the values obtained from cells incubated with empty vesicles. O——O: empty vesicles. ●——●: empty vesicles premixed with free RNase. ▲——▲: vesicles containing RNase; a, b and c designate 5, 10 and 20 µg enzyme per µmole PS, respectively. □——□: anisomycin plus puromycin. A: Zajdela cells; B: lymphosarcoma cells.

either RNase-A, RNase T_1, or a cross-linked dimer of RNase A (25). Some variations in vesicle lipid composition were also tried out: incubation of cells with vesicles containing 5 mole % lysoPC as a fusogen (26) led to rapid loss of cell viability; no effect resulted from incorporation of up to 40 mole % of cholesterol into PC/PS vesicles, nor did we observe any effect with vesicles composed of dioleoyllecithin. Arguing that the lack of effect of the enzyme could still be due to its inability to enter the cytoplasm, and bearing in mind the succesful efforts of others (12,14) in introducing "foreign" substances into cells using vesicles composed of pure PS, we recently incubated Zajdela cells and lymphosarcoma cells *in vitro* with RNase-A-containing small unilamellar vesicles, made up exclusively from PS. The first trials, which were carried out under similar experimental conditions (temperature, vesicle lipid concentration, cell density etc.) as were applied in our experiments indicated above, showed a dramatic effect of liposome-entrapped RNase A, as can be seen in Fig. 2. Both for Zajdela cells (Fig. 2.A) and lymphosarcoma cells (Fig. 2.B) the incorporation of leucine into *de novo* synthesized total cellular protein decreases progressively with incubation time and with liposomal RNase-concentration. Generally, the degree of inhibition is greater for lymphosarcoma cells than for Zajdela cells. With the lymphosarcoma cells free RNase also shows a slight inhibition of leucine-incorporation. Preliminary experiments indicate that the inhibition is directly correlated to the vesicle-lipid concentration in the incubation mixture. These results strongly suggest that the mode of interaction of PS-vesicles with cells is qualitatively and/or quantitatively different from that of vesicles composed of PC, cholesterol and PS (molar ratio 5 : 4 : 1) or of other compositions mentioned above. Further experiments are needed to fully understand the mechanism by which the observed effect of the liposome-entrapped enzyme is accomplished.

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S A M E N V A T T I N G

Dit proefschrift behelst een onderzoek naar een aantal aspecten van de interactie tussen liposomen en tumorcellen. Liposomen zijn op kunstmatige wijze gevormde,kleine partikels(diameter ca 25-2500 nm),opgebouwd uit één of meer concentrische bimoleculaire fosfolipide-membranen,welke evenzo vele waterige compartimenten omsluiten. De basisstructuur van liposomen,de fosfolipide-membraan,is dezelfde als die van in de natuur voorkomende cellulaire membranen(biomembranen). Vanwege deze strukturele overeenkomst worden liposomen in toenemende mate gebruikt als modelmembranen om de details van eigenschappen en functies van biomembranen te bestuderen.

Gedurende de laatste jaren is er tevens grote belangstelling gegroeid voor liposomen als "capsules". Allerhande moleculen kunnen op eenvoudige wijze in de liposomale membraan of in het inwendige waterige compartiment worden gehuisvest,en aldus in principe worden vervoerd naar cellen,zowel *in vitro* als *in vivo*. Onze belangstelling voor de interaktie tussen liposomen en tumorcellen kwam voort uit de door een aantal onderzoekers gesuggereerde mogelijke toepasbaarheid van liposomen als carriers voor cytostatisch werkzame geneesmiddelen bij de chemotherapie van kanker.

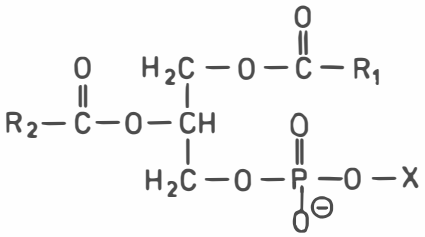
Het in dit proefschrift beschreven onderzoek heeft hoofdzakelijk betrekking op de wijze en mate van *in vitro* interaktie tussen uit rattelever-parenchym stammende Zajdela ascites hepatoma cellen en kleine unilamellaire lipide vesicles (=liposomen), opgebouwd uit phosphatidylcholine, cholesterol en phosphatidylserine. Na een beknopte inleiding over de struktuur, eigenschappen en toepasbaarheid van liposomen, wordt in twee experimentele hoofdstukken aandacht besteed aan de kinetiek en mechanismen van bovengenoemde interaktie. Met behulp van radioactief gemerkte lipiden in de liposomale membraan en in de liposomen ingesloten fluorescerende verbindingen wordt aangetoond dat liposomen hun inhoud op het inwendige van de cel kunnen overdragen via een membraanfusie-achtig proces. Tevens worden aanwijzingen verkregen dat er naast deze overdracht-komponent een "adsorptie"-komponent bestaat: liposomen kunnen waarschijnlijk min of meer passief binden aan het celoppervlak; daarbij lijkt hun inhoud vrij te komen in het omgevende medium. Het derde experimentele hoofdstuk gaat nader in op deze door de cel-oppervlakte geïnduceerde lekkage. Dit fenomeen blijkt o.a. samen te hangen met het cholesterol-gehalte van de liposomen,

en wordt waarschijnlijk veroorzaakt door interaktie van de liposomen met celoppervlakte-eiwitten. Het vierde hoofdstuk beschrijft de resultaten van een peilstudie naar de bruikbaarheid van liposomen als transportmiddel *in vivo* voor cytostatische geneesmiddelen. Aangetoond wordt dat verpakking van het cytostaticum 1-β-D-arabinofuranosyl-cytosine in liposomen na intraperitoneale injektie bij de muis tot een verhoogde remming van de groei van een lymphosarcoom in de milt leidt, mogelijk als gevolg van een combinatie van langzaam vrijkomen van het geneesmiddel uit de liposomen en een direkte interaktie tussen liposomen en tumorcellen. Met behulp van een tweetal- metabool inerte- in liposomen ingesloten stoffen wordt vastgesteld, dat liposomen na intraperitoneale injektie o.a. in de tumordragende milt terechtkomen. Het vijfde experimentele hoofdstuk beschrijft dunnelaaggelchromatografie als een nieuwe methode, waarmee op eenvoudige en snelle wijze een indruk omtrent de gemiddelde grootte van liposomen verkregen kan worden. In het laatste hoofdstuk wordt een werk-hypothese voor verder onderzoek naar het mechanisme van liposoom-cel interaktie opgesteld. De aandacht wordt gevestigd op de betekenis die aan fusie tussen liposomale - en plasmamembraan zou kunnen worden toegedacht bij de overdracht van in liposomen ingesloten stoffen op het inwendige van de cel. Het hoofdstuk wordt besloten met de beschrijving van een aantal pogingen om met behulp van een in liposomen ingesloten enzym, ribonuclease, het eiwitsynthetiserend apparaat in het cytoplasma van Zajdela cellen en lymphosarcoma cellen te attaqueren.

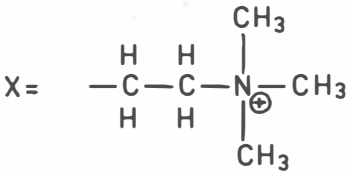
LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di(3-ethylbenztiazolin)-sulphonic acid
AECM	N-(2-aminoethyl)carbamylmethyl-
approx.	approximately
Ara-C	1-β-D-arabinofuranosyl-cytosine
a.u.	arbitrary unit
BHK	baby hamster kidney
BSS	basal salt solution
CF	carboxyfluorescein
Chol.	cholesterol
Chol. ol.	cholesterol oleate
Ci	Curie
DCP	dicetylphosphate
diI	$3,3^1$-dioctadecylindocarbocyanine
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylene diamine tetraacetate
F	fluorescence
FDX	FITC-tagged dextran
FITC	fluorescein-isothiocyanate
FRAP	fluorescence recovery after photobleaching
g	force of gravitation
h	hour
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
HRPO	horse radish peroxidase
LDH	lactate dehydrogenase
Leu	leucine
LS	lymphosarcoma
mol(e)	mole
Me	methyl-
μM	micromolar (10^{-6} moles. $liter^{-1}$)
mM	millimolar (10^{-3} moles. $liter^{-1}$)
min	minute
M	molar (moles. $liter^{-1}$)
MLV	multilamellar vesicle

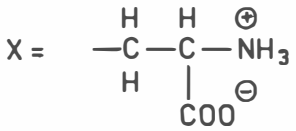
M.R.	molar ratio
mRNA	messenger RNA
M.W.	molecular weight
NAN	N-acetyl-neuraminosyl-
NBD-PE	N-4-nitrobenzo-2-oxa-1,3-diazole-phosphatidylethanol-amine
nm	nanometer (10^{-9} meter)
PC	phosphatidylcholine
pmol(e)	picomol(e) (10^{-12} mole)
PS	phosphatidylserine
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
s	second
S.D.	standard deviation
SUV	small unilamellar vesicle
TLC	thin layer chromatography
Tris	tris (hydroxymethyl)aminomethane
v/v	volume/volume
w/v	weight/volume



Basic structure of 3-*sn*-phosphoglycerides. R_1 and R_2 represent the hydrocarbon moieties of the fatty acyl chains.



phosphatidylcholine (lecithin)



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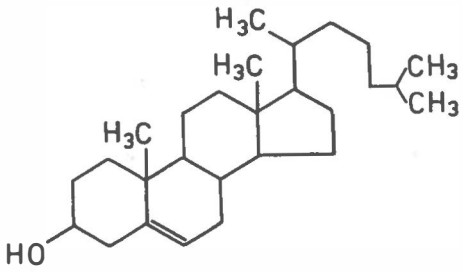
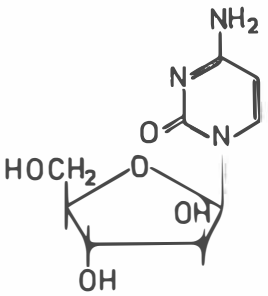
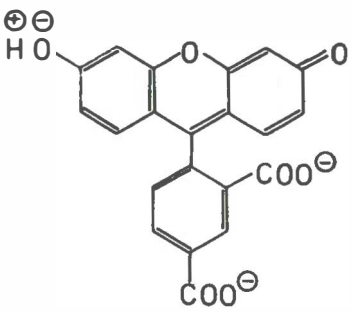


cholesterol



1-β-D-arabinofuranosyl-cytosine



carboxyfluorescein